Prevalence and Load of Airway Bacteria in Chronic Obstructive Pulmonary Disease

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I, Davinder Singh Garcha, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis

Abstract

Chronic obstructive pulmonary disease (COPD) is defined by irreversible airflow limitation, usually caused by exposure to noxious particles or gases. COPD patients suffer from chronic daily symptoms, and may occasionally suffer acute exacerbations – episodes in which there is a worsening of symptoms above day-to-day levels. Exacerbation aetiology is variable and controversial, although infection and air pollution are believed to play a part. Certain bacterial pathogens, known as typical airway bacteria, are found with high prevalence in individuals at exacerbation. The burden of other airway bacteria is currently ill-defined.

This study initially compared microbiological culture with quantitative PCR for detection of three commonly identified airway bacteria in COPD, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis* (typical airway bacteria). Quantitative PCR was utilised to establish prevalence and load of these species at stable, exacerbation and exacerbation recovery states. Typical airway bacterial prevalence and load was assessed against a range of clinical factors in COPD. Additional quantitative PCRs examined the prevalence and load of atypical airway bacteria (*Chlamydophila pneumoniae*, *Legionella pneumophila*, *Mycoplasma pneumoniae*) and the entire bacterial microflora of the lungs.

My original contribution to knowledge is that higher load of typical airway bacteria is associated with higher levels of systemic inflammation and airflow limitation in both stable and exacerbated COPD. For the first time it has also been demonstrated that airway microbiome load is not associated with airflow limitation or systemic inflammation changes, providing evidence that typical airway bacteria in particular are contributing to disease severity. Atypical airway bacteria prevalence in COPD was negligible.

The investigations in this thesis highlight the need for rapid antibiotic therapy in exacerbations where typical airway bacteria presence is suspected or confirmed. Furthermore, prophylactic antibiotic therapy should be considered for stable COPD patients with confirmed typical airway bacterial presence, as a means of reducing inflammation and airflow limitation.

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Abbreviations

AAT = α1-antitrypsin

AE-COPD = Acute exacerbations of chronic obstructive pulmonary disease

BAL = Broncho-alveolar lavage

CAP = Community-acquired pneumonia

CF = Cystic fibrosis

CFTR = Cystic Fibrosis Transmembrane Conductance Regulator

CFU = Colony forming units

ChoP = Phosphorylcholine

COPD = Chronic obstructive pulmonary disease

C. pneumoniae = *Chlamydophila pneumoniae*

cps = Capsular polysaccharide layer

CRP = C=reactive protein

CXCL-10 = C-X-C motif chemokine 10

FEV_1 = Forced expiratory volume in one second

FVC = Forced vital capacity

GOLD = Global Initiative for Chronic Obstructive Lung Disease

GSH = Glutathione

HDAC2 = Histone deacetylase-2

H. influenzae = *Haemophilus influenzae*

IAC = Internal amplification control

ICS = Inhaled corticosteroid

IκBα = inhibitory protein κBα

IKK = IκB-kinase

IL-1α = Interleukin-1α

IL-8 = Interleukin-8

LABA = Long-acting β-2 adrenoceptor agonist

LABC = Lower airway bronchial colonisation

LAMA = Long-acting muscarinic antagonist

L. pneumophila = *Legionella pneumophila*

LPS = Lipopolysaccharide

LRT = Lower respiratory tract

LRTI = Lower respiratory tract infection

LTB4 = Leukotriene B4

M. catarrhalis = *Moraxella catarrhalis*

MM = Mastermix

MMP = Matrix metallo-protease

M. pneumoniae = *Mycoplasma pneumoniae*

NAAT = Nucleic acid amplification test

NF-κB = Nuclear factor-κB

NICE = National Institute of Health and Clinical Excellence

NTHi = Non-typeable *Haemophilus influenzae*

PavA = Pneumococcal adhesion and virulence factor A

PCR = Polymerase chain reaction

PSB = Protected specimen brush

PspA = Pneumococcal surface protein A

qPCR = Quantitative polymerase chain reaction

RSV = Respiratory syncytial virus

SABA = Short-acting β-2 adrenoceptor agonist

SAMA = Short-acting muscarinic antagonist

SERPIN = Serine protease inhibitor

SNP = Single nucleotide polymorphism

S. mitis = *Streptococcus mitis*

S. pseudopneumoniae = *Streptococcus pseudopneumoniae*

S. pneumoniae = *Streptococcus pneumoniae*

TNF-α = Tumour necrosis factor-α

URT = Upper respiratory tract

Publications & Abstracts

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CHAPTER 1. **Introduction**

1.1 Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is a heterogeneous disease, made up of a number of different syndromes which are defined as COPD through spirometry testing. The most widely accepted current definition for COPD is that outlined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD), in which COPD is defined as "a preventable and treatable disease with some significant extrapulmonary effects that may contribute to the severity in individual patients. Its pulmonary component is characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases" (Rabe et al. 2007). This definition encompasses a wide variety of diseases, ranging from chronic bronchitis to emphysema (Polkey 2008).

Chronic bronchitis is defined clinically as the presence of cough and sputum production for three months per year for at least two successive years (Baik et al. 2008). Conversely, the definition of emphysema is pathological, by enlargement of the airspace and concomitant destruction of alveolar septae (Horowitz et al. 2009).

In accordance with National Institute of Health and Clinical Excellence (NICE) guidelines in the UK, diagnosis of COPD is considered in patients over the age of 35 who have symptoms of chronic cough, regular sputum production or exertional breathlessness, or a history of exposure to risk factors for the disease (Bellamy 2004). Diagnosis is clinically confirmed via spirometry testing, and mirrors GOLD guidelines (www.goldcopd.org) (Table 1.1). Spirometry testing involves the patient

taking a deep breath and exhaling as hard and fast as possible into a spirometer. The amount of air expired in the first second following expiration is recorded – this is known as forced expiratory volume in one second (FEV_1). Also measured is the forced vital capacity (FVC) – this quantifies the time taken to expire all air from the lungs.

Stage	Disease status	FEV_1/FVC	FEV_1
I	Mild	<0.70	≥80% predicted
II	Moderate	<0.70	50%≤FEV_1<80% predicted
III	Severe	<0.70	30%≤FEV_1<50% predicted
IV	Very severe	<0.70	FEV_1<30% predicted

Table 1.1. NICE COPD severity classification based on spirometry FEV_1 measurement. FEV_1: Forced Expiratory Volume in one second; FVC: Forced Vital Capacity.

COPD is a major global health burden, having a significant impact in terms of both morbidity and mortality. In 2010, COPD was the third biggest cause of death globally (Lozano et al. 2013). The major risk factor associated with development of COPD is tobacco smoking, particularly in high-income countries, where 73% of deaths from COPD are related to tobacco smoking (van Zyl Smit et al. 2010). This compares to low- and middle-income countries, in which only 40% of COPD mortality is related to tobacco smoking, with an additional 35% mortality attributable to exposure to biomass fuel smoke (van Zyl Smit et al. 2010), where biomass fuel refers to fuel derived from living/ previously living organisms.

It is unclear precisely how many smokers develop COPD, although estimates range from 15% to 50% (Polkey 2008; Laniado-Laborín 2009). Given that not all smokers develop COPD - regardless of their smoking history – it would appear that an abnormal inflammatory response to noxious particles may play a significant role in the development of the disease. In a lung health study of 4194 subjects with airflow obstruction, it was reported that 52.2% were smokers (Anthonisen et al. 2002). It should also be noted that in a small subset of COPD patients, α1-antitrypsin (AAT) deficiency rather than exposure to noxious particles is considered the principal factor in disease progression (Abboud & Vimalanathan 2008).

Patients with COPD usually exhibit a state which involves chronic symptoms, with little day-to-day variation. This typically involves being on regular maintenance therapy, and is described as stable COPD (NICE 2004). However, occasionally, patients suffer from an acute worsening of symptoms, often requiring additional therapy – this is known as an acute exacerbation of COPD (www.goldcopd.org).

1.1.1. COPD: A historical perspective

Some of the earliest known writings on COPD come from the 17th century, with a description from Bonet of voluminous lungs (Petty 2006). In 1814, the British doctor Charles Badham was one of the first individuals to recognise and highlight the chronic bronchitis component associated with COPD (Badham 1814). Less than a generation following this description, the French physician René Laennec found that some patients with obstructive lung disease had hyperinflated lungs which did not empty well (Laennec 1829). This finding has proven to be of extreme

importance in COPD diagnosis, as it is investigated in the form of lung spirometry testing. The spirometer was initially developed in 1846 by John Hutchinson, but it was only in 1947 that it was adapted to include a timed measure of FVC as we are now familiar with it (Hutchinson 1846; Tiffeneau & Pinelli 1947).

1.1.2. Aetiology of COPD

Tobacco smoking is widely acknowledged as the principal cause of COPD, with other causes including biomass fuel, air pollution and chemical fumes. However, not all long-term smokers go on to develop COPD, with estimates of COPD amongst smokers ranging from 15-50% (Fletcher & Peto 1977; Lundback et al. 2003). This indicates a genetic susceptibility towards the development of COPD. A well-defined phenotype in such development is AAT deficiency. This leads to a lack of protection in the lung against proteolytic enzymes, as AAT is responsible for neutralising these proteases, most notably neutrophil elastase (Stoller & Aboussouan 2012). This can ultimately result in the emphysematous subtype of COPD, both in smokers and non-smokers with the deficiency. However, this is a relatively rare deficiency, and it is responsible for COPD in only 1-2% of patients (Chitkara & Hurst 2012; DeMeo & Silverman 2004).

1.1.3. History of COPD identification

The heterogeneic nature of COPD gave rise to difficulties in terms of defining the disease, and this became a particular problem several decades ago, as cases of

pulmonary disease were increasing. In the 1950s and 1960s two rival hypotheses emerged. One of these became known as the Dutch hypothesis. This proposed that airway obstruction such as that seen in asthma, emphysema and chronic bronchitis are different phenotypes of one disease entity, with a strong genetic component termed chronic non-specific lung disease (Orie & Sluiter 1961). An alternative hypothesis, termed the British hypothesis, suggested that chronic bronchitis predisposed the lungs to subsequent infection, leading to damage to either the airways or alveoli, or both, triggering progressive airflow limitation (Stuart-Harris et al. 1953). Given the heterogeneous nature of COPD aetiology and progression, it is possible that both hypotheses may be correct.

Chronic bronchitis and emphysema are distinct conditions with regards to how pulmonary disease arises in either case, but are frequently termed together as COPD, and the reason for this is that that they share the phenotype of irreversible obstruction to bronchial airflow (Fletcher & Peto 1977). The two conditions therefore represent opposite poles of the COPD disease 'spectrum'. It is now recognised that the vast majority of COPD patients fall somewhere in the middle of the spectrum, exhibiting symptoms of both conditions (Mannino & Buist 2007).

1.1.4. Cigarette smoke, genetics and COPD

Chronic airway inflammation is a ubiquitous feature of COPD. The initial stimulus for such inflammation is noxious gas inhalation, normally through cigarette smoke.

Smoking triggers oxidative stress in the lungs, which is thought to be a result of oxidant-antioxidant imbalance (MacNee 2000). Furthermore, smoking also causes neutrophil infiltration (Baraldo et al. 2004). These neutrophils release elastase, a protease which contributes to the degradation of elastin in the lungs, and this is thought ultimately to lead to emphysema (Houghton et al. 2006).

Cigarette smoke is made up of a mixture of more than 4,700 chemicals, with more than 10^{17} molecules present in each puff (MacNee 2000). Oxidative stress is triggered by smoking through two mechanisms: cigarette smoke itself contains oxidants such as free radicals (10^{14} per puff), nitric oxide and nitrogen dioxide (300 to 500 parts per million) (Rahman et al. 1996). Furthermore, cigarette smoke induces tissue to produce oxidants (Barreiro et al. 2010), further contributing to high levels of oxidants in the lungs.

In the body, oxidants are counteracted by antioxidants, such as glutathione (GSH), vitamin C and vitamin E, both intra- and extra-cellularly (MacNee 2005). These antioxidants act as reducing agents, removing the danger of oxidative stress. In COPD, this balance between oxidants and antioxidants is believed to be circumvented, resulting in an emphysematous phenotype.

It is known that patients with COPD have higher levels of oxidative stress, relative to both healthy subjects and smokers without COPD (MacNee 2005). It is logical that oxidative stress would be higher in COPD than in healthy subjects, given that cigarette smoke introduces so many oxidants. However, to understand why

smokers without COPD have lower levels of stress than COPD patients, the impact of the disease must be considered.

As well as cigarette smoke itself, in COPD there are a number of other mechanisms which contribute to oxidative stress, particularly those in relation to chronic airway inflammation. This is driven by neutrophils, which are major secretors of reactive oxygen species, such as myeloperoxidase (Rahman & Kinnula 2012).

Elastin is a key protein of skin, blood vessels and lung architecture. In the lungs, it is part of the elastic fibre make-up, conferring the ability for the lungs to stretch and undergo elastic recoil (Turino et al. 2011). COPD patients with an emphysematous phenotype present disrupted elastic fibres. This is due to the action of proteases in degrading elastin. In healthy subjects, the proteolytic action of such enzymes is mitigated by the action of anti-proteases, including AAT.

Smoking is known to induce recruitment of high numbers of neutrophils to the airways (Hunninghake & Crystal 1983). During an inflammatory response, neutrophils secrete neutrophil elastase, which is capable of destroying both bacteria and elastin. AAT is able to combat such degradation, and is also able to inhibit apoptosis, by preventing the action of executioner caspases. However, cigarette smoke acts directly to inactivate methionine residues of AAT, causing a loss of anti-elastase activity (Taggart et al. 2000).

Matrix metallo-proteases (MMPs), secreted by alveolar macrophages, are also associated with degradation of lung tissue, including elastic fibres and collagen (Russell et al, 2002). Various MMPs, including MMP1, MMP2, MMP9 and MMP12 are thought to be involved, where patients with 'unfavourable' allele polymorphisms are more susceptible to the effects of smoking (Mocchegiani et al. 2011).

It has been noted that oxidative stress causes oxidative inactivation of antiproteases (MacNee 2000). This indicates that the oxidant-antioxidant imbalance and the protease-antiprotease imbalance may be interlinked.

1.1.5. Genetics and COPD

In recent years, increasing attention has been given to the role that genetics may play in pre-disposing certain individuals to developing COPD. It has previously been described that presence of COPD appears to aggregate familially, independent of gender, smoking history and presence of AAT deficiency (Tager et al. 1978).

AAT is produced by hepatocytes in the liver and is normally present in the serum at a concentration of between 20-53 μM (Stoller & Aboussouan 2012). The active site of AAT binds to elastase, thereby resulting in its permanent inactivation (DeMeo & Silverman 2004). It has been found that four distinct AAT phenotypes exist in the general population: normal alleles; deficiency alleles; null alleles; dysfunctional

alleles. Normal alleles reflect the most prominent phenotype in the population, found in more than 95% of the Caucasian population (DeMeo & Silverman 2004). Null phenotypes have no AAT presence detected in serum, and carriers of this phenotype are at particularly increased risk of acquiring emphysema (Luisetti & Seersholm 2004).

As there is a familial linkage to COPD, independent of AAT deficiency, it is important to consider which other factors may be involved in this association. In a study which investigated COPD in families with no presence of AAT deficiency, where the proband demonstrated severe, early-onset COPD, it was identified that first-degree relatives of such probands had significantly lower FEV_1 and FEV_1/FVC ratio compared with controls (Silverman et al. 1998).

AAT is also known as SERPINA1 (*ser*ine *p*rotease *in*hibitor, Group *A*, Member *1*) (Schmechel 2007). Other SERPINs are also thought to be involved in protecting against proteolytic damage. Investigations have therefore been performed to assess whether dysfunction in these protease inhibitors also contributes to COPD development and progression. Augmentation therapy with AAT supplementation has previously been examined as a means of reducing bronchial inflammation in patients with AAT deficiency (Stockley et al. 2002). Stockley and colleagues found that augmentation therapy increased levels of serum AAT above the protective threshold and was associated with a decrease in both elastase activity and levels of the chemoattractant leukotriene B4 (LTB4).

Single nucleotide polymorphisms (SNPs) of the metalloprotease protein encoded by the gene *ADAM33* have been shown to be associated with COPD in long-term smokers, in a study involving non-COPD long-term smokers as controls (Sadeghnejad et al. 2009). This protein has previously been implicated in airway remodelling in asthma (Nelson et al. 2003) and the study by Sadeghnejad and colleagues therefore suggests a genetic mechanism by which some long-term smokers may develop COPD.

1.1.6. Inflammation

COPD is a disease in which the inflammatory response plays a major role in the manifestation of disease symptoms. This may be either directly, through airway inflammation, or indirectly through systemic inflammation. In studies examining inflammation in COPD, biomarkers of airway or systemic inflammation are typically measured for quantification.

As was previously described in section 1.1.4, cigarette smoking triggers airway inflammation. In a randomised cross-over study, van der Vaart and colleagues found that smoking increased levels of sputum neutrophils and interleukin-8 (IL-8) release in a group of healthy intermittent smokers (van der Vaart et al. 2005). Interestingly, in the same study it was found that smoking suppressed the levels of blood eosinophils (although the level of sputum eosinophils was not affected).

IL-8 is a chemokine produced in response to oxidative stress, and is involved in the recruitment of polymorphonuclear leukocytes, such as neutrophils (Eckmann et al.

1993). Neutrophils secrete elastase principally as a means to degrade bacterial cell membranes. However, elastase contributes to the degradation of elastin tissue in the lung parenchyma as well as mucous gland hyperplasia and mucus hypersecretion (Hoenderdos & Condliffe 2013). Furthermore, it reduces ciliary beat frequency, activates complement and inactivates immunoglobulin (Hoenderdos & Condliffe 2013). Interleukin-1α (IL-1α) has also been shown to be important for initiation of smoke-induced neutrophilic inflammation (Botelho et al. 2011).

Mucus hypersecretion involves release of the mucins MUC5AC and MUC5B, triggered by this secreted elastase (Park et al. 2005). Mucin is secreted by specialised cells of the airway epithelium and submucosa gland cells, and combines with other secretions including water, proteins and salt, to form mucus (Kirkham et al. 2008). From a clinical viewpoint, mucus hypersecretion is a common symptom in COPD, particularly in patients who exhibit the chronic bronchitic phenotype of chronic sputum production (Kim & Criner 2013).

Bafadhel and colleagues have previously demonstrated that different inflammatory biomarkers are found with differing clinical phenotypes, with IL-1β associated with bacterial exacerbations and serum CXCL-10 associated with viral exacerbations (Bafadhel et al. 2011).

Nuclear factor-κB (NF-κB) is a transcription factor which has an essential role in modulating the immune response to infection (Pasparakis 2012). It is able to control the expression of a wide variety of inflammatory and apoptosis-inducing

genes (Brown et al. 2009). Under non-stimulated conditions, NF-κB is inactive, bound to inhibitory protein κBα (IκBα) in the cytoplasm (Szulakowski et al. 2006). Activation occurs through cell signalling pathways: External stimuli such as cytokines or virulence factors trigger activation of IκB-kinase (IKK). IKK is then able to phosphorylate IκBα resulting in the latter's degradation (Karin 1999). This allows the unbound NF-κB to migrate to the nucleus and initiate transcription of a variety of genes involved in a pro-inflammatory response (Figure 1.1).

In healthy individuals, regulation of NF-κB is tightly controlled, ensuring that any inflammatory outcome does not have a detrimental impact to the host. In COPD, however, expression of phosphorylated NF-κB is significantly upregulated compared to the rates seen in both asymptomatic smokers and non-smoking controls (Aoshiba et al. 2012).

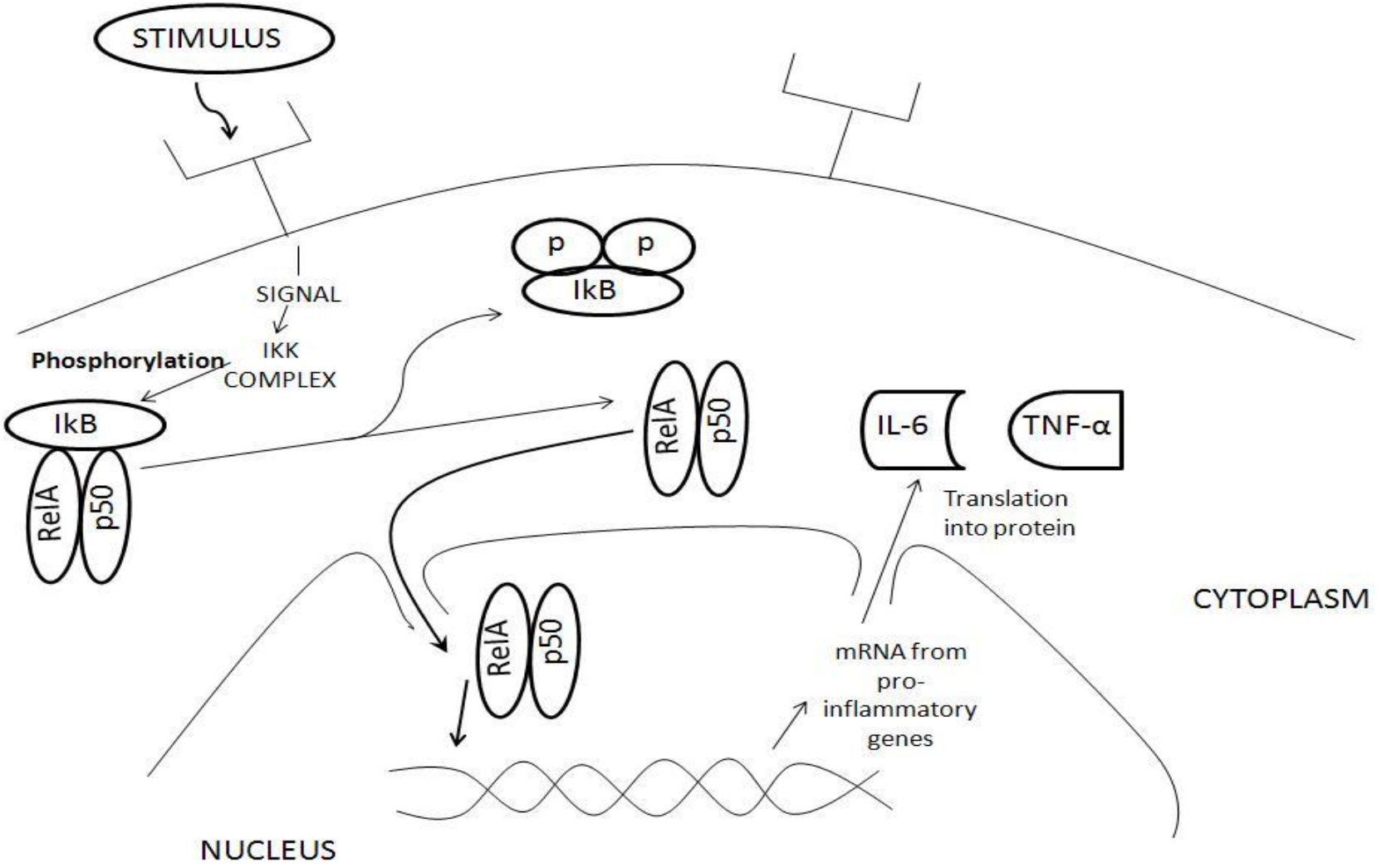


Figure 1.1 Cellular mechanism for activation of NF-κB. Abbreviations: IκB=inhibitor of kappa B; IL-6= interleukin-6; p=phosphate group; TNF-α=Tumour necrosis factor-alpha

Recent evidence, however, contradicts the view that cigarette smoking is a major driver of NF-κB-associated inflammation (Rastrick et al. 2013). Rastrick and colleagues examined the NF-κB:DNA binding activity in mouse lung extracts. They found no increase in NF-κB:DNA binding activity following exposure to cigarette smoke extract. However, they acknowledge that the time points at which they measured such activity may not have captured any increase – the earliest time point at which activity was measured was 2 hours post-challenge, by which time an acute inflammatory response may have dissipated.

In the present thesis, the association of inflammation and lower airway bacterial presence is considered in Chapters 4, 5, 6 and 7.

Contrary to the inflammatory phenotype identified in asthma, in which an eosinophilic influx is commonly seen, neutrophil recruitment is a routine component of the inflammatory response triggered in COPD (Stockley 2002). This contributes significantly to the emphysematous phenotype seen in many cases of COPD due to the excessive production of neutrophil elastase, as discussed in previous sections. Understanding the basis of neutrophilic infiltration is therefore an important step to understanding inflammation in COPD.

Neutrophils migrate towards sites of inflammation via chemotaxis, directed by chemokines (Bracke et al. 2007). LTB4 is a chemoattractant known to play a major role in the recruitment and activation of neutrophils (Afonso et al. 2012). Afonso and colleagues found that it is secreted by activated neutrophils as they migrate

towards the site of inflammation. This generates a signalling-relay, amplifying neutrophil recruitment. In COPD, levels of LTB4 are significantly higher than those in healthy controls (Profita et al. 2010).

1.1.7. Differences between healthy smokers and COPD patients

Whilst smoking is known to be the most common risk factor associated with COPD, not all smokers will develop the disease. Given this discrepancy between healthy smokers and smokers with COPD, and in order to understand the nature of the disease, it is therefore of importance to examine the factors that may pre-dispose certain people to be more susceptible than others.

Schulz and colleagues examined rates of various inflammatory modulators in COPD patients versus asymptomatic smokers, with eight subjects examined in each group (Schulz et al. 2004). They found that both IL-8 and the related chemokine GRO-α increased at a significantly higher rate in COPD patients than in the control group, following stimulation with tumour necrosis factor-α. Additionally, COPD patients have higher neutrophil levels compared with non-COPD smokers (Keatings et al. 1996).

1.1.8. Pathology

It is known that structural and functional changes in the small airways (<2mm diameter) result in airflow limitation in COPD (Sturton et al. 2008). Chronic inflammation in the lungs triggers fibrosis (Wick et al. 2013). Furthermore, loss of

alveolar attachments may be identified, also due to inflammation (Saetta et al. 1985). In healthy individuals, alveolar attachments ensure that airways do not fully close during expiration. Elastin fibres in the alveolar attachments regulate this process. However, in COPD elastin is degraded. A potential consequence of this is that small airways without alveolar attachments may close completely, limiting expiration of air from the lungs, and causing air trapping. Air trapping is associated with hyperinflation of the lungs, and this leads to increased levels of dyspnoea and reduced exercise tolerance (Gibson 1996).

It has also previously been elucidated that small airways inflammation is correlated with disease severity (Hogg et al. 2004). This would support the theory of a vicious circle involving disease severity and inflammation (Figure 1.2).

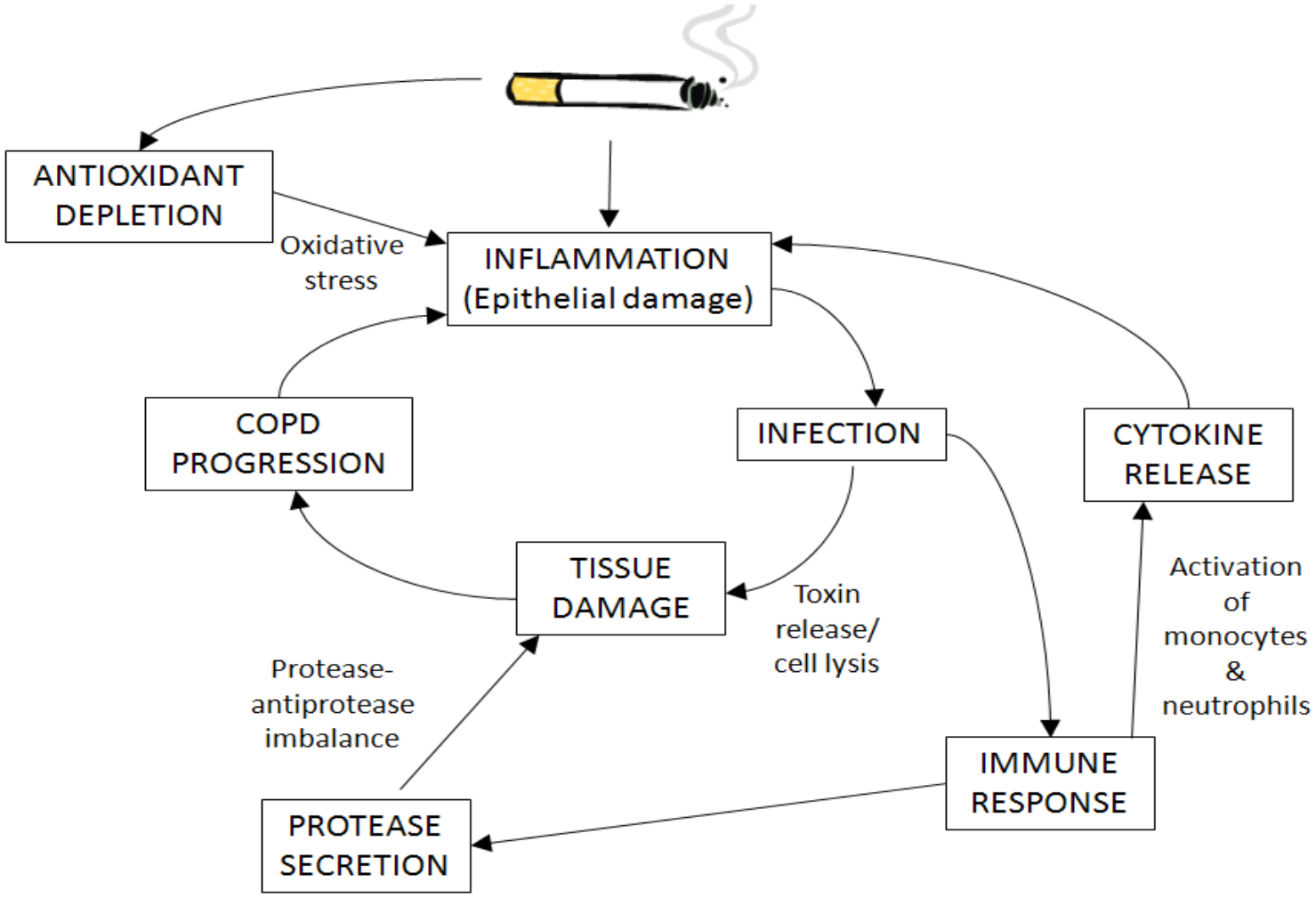


Figure 1.2 The vicious circle hypothesis of inflammation in COPD

1.1.9. Treatment of stable COPD

There is no known cure for COPD. However, there are a variety of therapies available by which symptoms can be alleviated. The single most effective treatment for COPD is smoking cessation (Anthonisen et al. 1994; Kanner et al. 1999). Ever since the seminal paper by Fletcher and Peto in 1977 examining the effect of smoking cessation in patients with COPD, it has been known that stopping smoking can dramatically reduce the rate of decline in FEV_1, compared with those patients who continue to smoke (Fletcher & Peto 1977). Nonetheless, with or without smoking cessation, disease progression continues to some extent and so therapy is usually required.

During stable COPD, a common treatment is bronchodilator therapy, via inhalation. Such treatment is principally in the form of β-2 adrenoceptor agonists, and this may be short-acting (SABA), e.g. Salbutamol, or long acting (LABA), e.g. Salmeterol. Occasionally, long-acting and short-acting muscarinic antagonists (LAMA/SAMA) are used alternatively, or in combination with β-2 agonists. SABAs have a rapid effect (within 5 minutes), with a total duration of between 4-6 hours (Beeh & Beier 2010) As such, they are often taken as reliever medication during periods of breathlessness. LABAs begin to act after 20 minutes, lasting for a total of 12 hours (Decramer et al. 2013). They are utilised as maintenance therapy and taken twice-daily by COPD patients.

SAMAs, such as ipratropium bromide, are provided as nebuliser therapy and act within 15 minutes of use, for a total of 4-6 hours (Noord et al. 2000). Conversely, the LAMA, tiotropium, can be taken as a once-daily medication (Noord et al. 2000). In a recent meta-analysis comparing use of tiotropium or placebo, it was identified that use of tiotropium was significantly associated with improvements in lung function, quality of life and exacerbation risk (Karner et al. 2012).

The two bronchodilator therapies have different mechanisms of action: β-2 agonists target the β2-adrenergic receptors present on smooth muscle, causing relaxation of such muscle, with resultant dilation of bronchi (Cazzola et al. 2013). Anti-cholinergic therapies work by inhibiting the activity of acetylcholine receptors, thereby preventing bronchial constriction (Cazzola et al. 2012).

A recent development in COPD treatment is the discovery of ultra-long acting β-agonists, such as carmoterol and indacaterol, paving the way for once-daily dosing (Beeh & Beier 2010). In a randomised, double-blinded crossover trial comparing indacaterol with tiotropium (a LAMA) and a placebo, it was found that indacaterol provided significant bronchodilation over a 24-hour period, at least as effective as tiotropium (Vogelmeier et al. 2010). This finding was supported by a recent pooled analysis examining the efficacy of indacaterol in a number of different clinical trials – this analysis agreed that indacaterol was at least as effective as tiotropium, and also found that it was better than both salmeterol and formoterol (Yorgancioglu 2012).

Where the clinician deems necessary, inhaled corticosteroids (ICS) may also be prescribed to reduce airway inflammation. ICS are frequently used in airway diseases in order to reduce inflammation (Confalonieri et al. 1998). However, they are generally regarded as having greater potency in other airway diseases such as asthma, compared to their effectiveness in COPD (Barnes 2006). Nonetheless, ICS are considered an integral part of COPD therapy.

In a systematic review, it was demonstrated that ICS treatment reduces exacerbation frequency (Alsaeedi et al. 2002). However, in the same analysis, it was also demonstrated that there were a number of side-effects associated with ICS use. As such, the suitability of ICS use at stable state must be carefully considered. The ISOLDE trial investigated the efficacy of the ICS, fluticasone propionate, compared with a placebo. It was found that ICS treatment reduced exacerbation frequency and also slowed the rate of decline in health status (Burge 2000). In two major trials in which the effects of ICS in COPD were evaluated (TORCH and ECLIPSE trials), it was found that ICS usage was associated with a higher rate of pneumonia in COPD patients than that seen with other treatments (Calverley et al. 2007; Wedzicha et al. 2008; Calverley et al. 2011). This association needs further exploration on a mechanistic basis, and is examined in the present thesis in Chapter 5.

In a study investigating the efficacy of ICS/LABA combination therapy, involving patients with severe COPD, it was seen that there was a 35% reduction in

exacerbation frequency with the combination therapy, compared to use of a LABA alone (Kardos et al. 2007).

ICS therapy is commonly given in asthma treatment, and is considered highly effective at reducing inflammation in the disease. Conversely, in COPD, patients occasionally demonstrate insensitivity to ICS treatment. One potential reason for this discrepancy which has been mooted is the reduction in activity of histone deacetylase-2 (HDAC2) in COPD patients (Barnes 2004). HDAC2 is an enzyme which catalyses deacetylation of histones in chromatin, resulting in prevention of transcription occurring. However, in COPD, the consequence of reduced HDAC2 activity is a higher level of acetylation occurring, and this has been associated with increased transcription of the cytokine IL-8, contributing to persistent inflammation.

1.1.10. Lower airway bronchial colonisation

Lower airway bronchial colonisation (LABC) is a common feature of COPD. Conventional microbiological culture has previously demonstrated the presence of typical airway bacteria including *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Serological analysis has also linked atypical bacteria such as *Legionella pneumophila*, *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* to COPD exacerbations (Lieberman et al. 2002a; Lieberman et al. 2001; Lieberman et al. 2002b). Presence of lower airway bacteria is associated with higher airway

inflammation at both stable and exacerbation states of COPD (White et al. 2003; Soler et al. 1999).

It has been frequently demonstrated, using routine microbiological culture, that lower airway bacteria are associated with COPD, both in the stable state and at exacerbation - with the bacteria most commonly identified being *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Pseudomonas aeruginosa* (Rosell et al. 2005; Miravitlles et al. 1999). The study by Rosell and colleagues found prevalence of presence of *H. influenzae*, *M. catarrhalis* and *S. pneumoniae* at 30% during stable state and 44% during exacerbation state. These 'typical airway bacteria' are commonly associated with infection in COPD patients.

1.2 Acute Exacerbations of COPD

There is no generally agreed definition of acute exacerbations of COPD (AE-COPD) although it is associated with a worsening of COPD symptoms compared to usual daily symptoms (Burge & Wedzicha 2003). This can be measured according to a number of different criteria. According to Anthonisen and colleagues, exacerbations can be defined based on the symptoms caused. If a patient saw a deterioration in the major symptoms, dyspnoea, sputum purulence and sputum volume, this was described as a Type 1 exacerbation, whilst if only two of these symptoms were seen, it was noted as a Type 2 exacerbation. If one of these major symptoms was identified, in conjunction with either cough, wheeze or respiratory tract infection, then a Type 3 exacerbation was declared (Anthonisen et al. 1987). An alternative method of recording an exacerbation is to define it according to health care utilisation - an event which results in a therapeutic intervention (Tashkin 2010).

1.2.1. AE-COPD: Triggers and associations

There are a number of factors known to be associated with exacerbations. A previous review highlighted viruses, bacteria and airway irritants (e.g. pollutants) as being associated with AE-COPD (Wedzicha & Seemungal 2007).

Bacterial pathogens are commonly identified in the lower airways of patients suffering exacerbations, with *Haemophilus influenzae*, *Streptococcus pneumoniae, Pseudomonas aeruginosa* and *Moraxella catarrhalis* particularly prevalent (Rosell et

al. 2005; Papi et al. 2006). However, this association is complicated by the fact that some COPD patients appear to be chronically colonised with lower airway bacteria, even during the stable state of the disease.

The major viral pathogen seen during an exacerbation is rhinovirus, with other viruses involved including respiratory syncytial virus (RSV) and coronavirus (Seemungal et al. 2001). Rhinovirus infects the airway epithelial cells lining the upper and lower airways. This stimulates the secretion of various cytokines, including Interleukin-6 (IL-6), IL-8 and CXCL1 (Schneider et al. 2010). IL-6 has a number of roles in modulating the inflammatory response. For example, it is able to stimulate T-cell activation and promote recruitment of macrophages and neutrophils, with consequent pathogenesis (Rincon 2012).

Acute exacerbations are usually treated with oral steroids and/or antibiotic treatment, dependent upon the symptoms presented. For example, purulent and excessive sputum production is indicative of a bacterial infection, which would pre-dispose the patient to antibiotic therapy (Anthonisen et al. 1987).

There is no standardised measure of length of antibiotic treatment for COPD exacerbations, and a typical treatment course is 7-10 days, with a second course given if symptoms have not resolved. There are a number of different types of antibiotics which may be prescribed. In the London COPD cohort, β-lactam antibiotics are the most frequently prescribed, namely amoxicillin and co-amoxiclav

(a combination of amoxicillin and clavulanic acid), although fluoroquinolone, macrolide and tetracycline antibiotics are also occasionally prescribed.

It has previously been demonstrated that antibiotic usage during COPD exacerbations reduces the frequency of subsequent exacerbations, and also reduces the risk of mortality (Roede et al. 2009).

β-lactam antibiotics

β-lactam antibiotics inhibit synthesis of the peptidoglycan cell wall (Tomasz 1979). This means that they are effective specifically in environments where bacteria are actively replicating. Clavulanic acid is often given in combination with β-lactams, as it is a potent inhibitor of β-lactam-degrading β-lactamase enzymes produced by resistant bacterial species (Reading & Cole 1977).

Fluoroquinolones

Fluoroquinolones constitute a family of synthetic, broad-spectrum antibiotics first identified in the 1960s. The *modus operandi* of the antibiotics involves prevention of DNA replication, specifically by inhibiting the mode of action of the bacterial enzymes DNA topoisomerase II (alias DNA gyrase) and DNA topoisomerase IV (Hooper 1999). This inhibition stops the catalysis of bacterial DNA unwinding which would be a necessary first step in DNA replication.

Macrolides

Macrolide antibiotics have bacteriostatic properties, meaning that they inhibit bacterial reproduction. They act by binding to the bacterial 50S ribosome subunit, therefore inhibiting protein synthesis. Specifically, macrolides interact with the peptidyltransferase ring located in the 23S rRNA subunit section, preventing extension of the growing peptide chain (Franceschi et al. 2004).

Macrolides also possess anti-inflammatory properties, in addition to the reduction of inflammation imparted by bacterial elimination (Cameron et al. 2012). The

benefits of macrolides as an immuno-modulatory drug were illustrated in the 1990s, when it was shown to dramatically improve mortality rates in patients with diffuse pan-bronchiolitis (Kudoh et al. 1998).

There are a number of mechanisms by which macrolides are thought to act to facilitate an anti-inflammatory outcome - It has been demonstrated that macrolides decrease mucus hypersecretion (Tamaoki et al. 1995) and reduce levels of pro-inflammatory cytokines in patients with chronic airways disease (Oishi et al. 1994).

Macrolides such as erythromycin, clarithromycin and azithromycin are frequently used to treat exacerbating COPD patients, and trials have also demonstrated efficacy of macrolides in reducing exacerbation frequency, when given to stable COPD patients (Seemungal et al. 2008; Albert et al. 2011).

Tetracyclines

Like macrolides, tetracycline antibiotics also inhibit protein synthesis. However, tetracyclines act by inhibiting the 30S subunit of prokaryotic ribosomes (Griffin et al. 2010).

The bacterial targets of the major antibiotic groups currently in use for COPD are summarised in Figure 1.3.

Systemic corticosteroids are also prescribed, according to GOLD COPD guidelines (www.goldcopd.org). A meta-analysis examining the use of systemic corticosteroids in the treatment of COPD exacerbations showed that treatment improves airflow limitation, decreases the risk of relapse and may improve symptoms and decrease the length of hospital stay (Schweiger & Zdanowicz 2010).

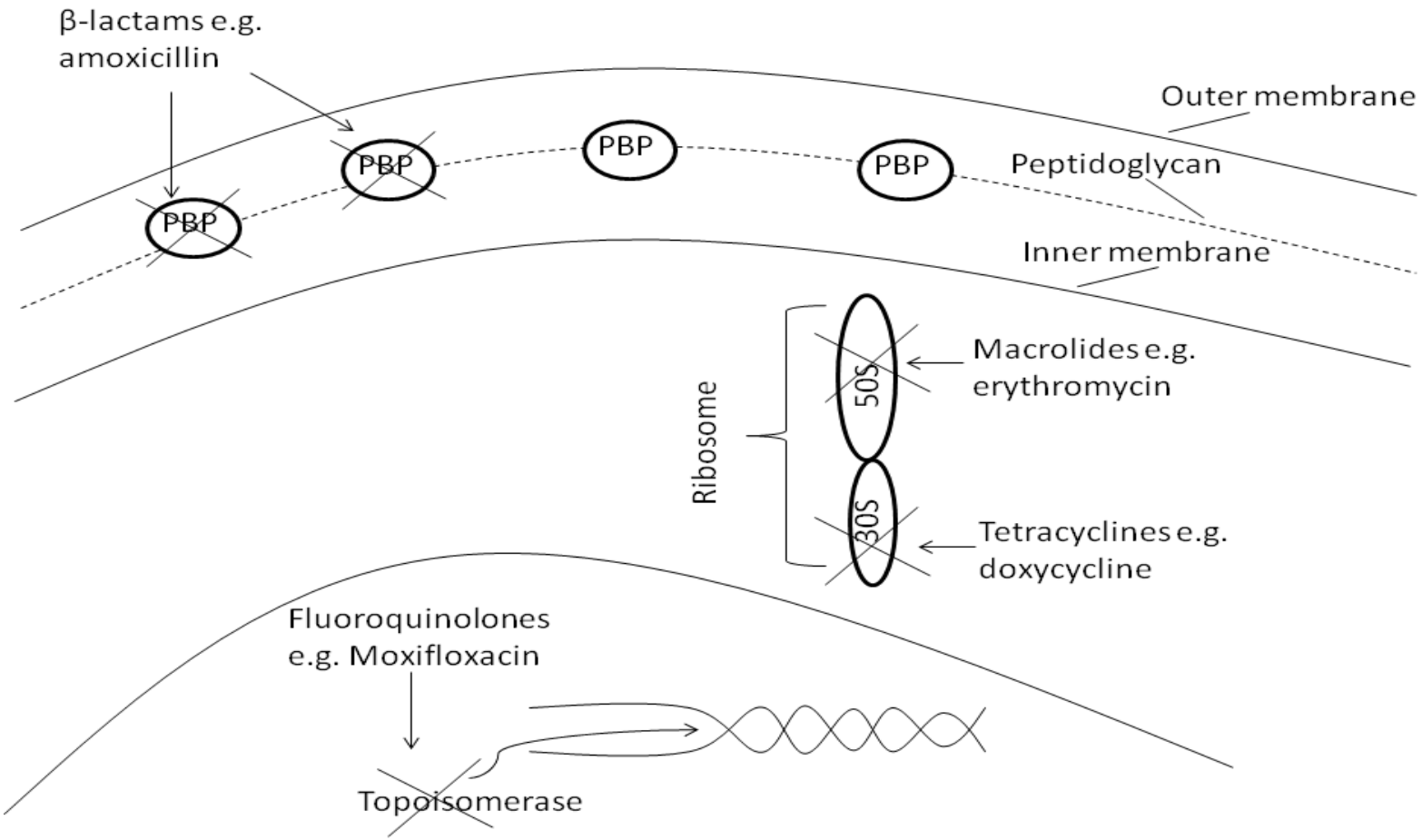


Figure 1.3. Antibiotic targets in a bacterial cell. PBP = Penicillin-binding protein

The relationship of antibiotic usage with development of antibiotic resistance has been previously well-characterised (Gould 1999). However, in the specific context for the treatment of COPD exacerbations, this relationship is not clearly defined.

Community-acquired pneumonia (CAP) is occasionally identified in COPD, with one study showing that 12-20% of COPD patients were diagnosed with pneumonia over a 3-year period (Calverley et al. 2007). CAP, which is distinct from AE-COPD, is also primarily caused by bacterial or viral infection of the lungs. In particular, *Streptococcus pneumoniae* has been found to be the aetiological agent in 48% of CAP cases in the UK, making it the single most important pathogen to target with antibiotic therapy (Lim et al. 2001).

It has been found that there is an association between use of ICS therapy and rate of pneumonia in COPD. Two randomised clinical trials, TORCH and INSPIRE, which examined the efficacy of ICS and combination therapies to treat COPD, both identified significantly increased rates of pneumonia in patients who had been treated with fluticasone compared to those treated with placebo (Calverley et al. 2007; Calverley et al. 2011). The mechanistic rationale for such a finding is not well understood, although it has previously been illustrated that the anti-inflammatory nature of ICS compromises the immune response (Adcock 2004).

1.3 Micro-organisms in COPD

The treatment of acute exacerbations of COPD with antibiotics has previously been considered. However, there is a growing body of evidence indicating that providing prophylactic antibiotic therapy to patients during the stable state may also prove beneficial in reducing the rate of exacerbations. In a Cochrane meta-analysis review, analysing nine trials performed before 1970, it was found that prophylactic antibiotic treatment in chronic bronchitis elicited a small, statistically significant effect in reducing days of illness caused by exacerbations (Staykova et al. 2003).

In a study involving the macrolide antibiotic erythromycin, our group has previously demonstrated that long-term macrolide treatment to COPD patients – 250mg twice daily for 12 months – significantly reduced exacerbation frequency versus placebo (rate ratio of 0.65 when placebo=1) (Seemungal et al. 2008). Whether this is due to the antibiotic or anti-inflammatory properties of macrolides (or a combination of both), is currently unclear.

Albert and colleagues built upon this research, treating 1142 COPD patients with 250mg daily azithromycin or placebo for 12 months (Albert et al. 2011). Again, it was seen that macrolide treatment significantly reduced exacerbation frequency (ratio of 0.73). They also noted a potential side effect whereby hearing decrements were higher in the azithromycin group (25% vs 20%).

The potential benefits of prophylactic antibiotic therapy have been highlighted by these recent studies, but work needs to be done to establish the implications of such treatment on the development of bacterial resistance.

1.3.1. London COPD cohort

The London COPD cohort is a prospective cohort study which has been established to investigate the causes and mechanisms of COPD exacerbations. In this study, a modified version of Anthonisen's criteria is utilised in which two or more symptoms appear or show deterioration, at least one of which must be a major symptom – sputum purulence, sputum volume, dyspnoea (Seemungal et al. 1998). Minor symptoms are cough, wheeze, sore throat and cold symptoms. The study involves a rolling cohort of patients and at any one time there are approximately 200 COPD patients in the study. These patients are able to record daily changes in physiology using a diary card based system (see Appendix). These diary cards record a number of parameters each day: Peak flow, Change in symptoms, Change in treatment, Hours out of the home, Number of steps.

Patients are seen quarterly for baseline visits, as well as during any reported exacerbations (patients are able to call a dedicated COPD phone held by a physician 24 hours a day, seven days a week). Each year, an annual review is performed, to update patient data. Following notification of an exacerbation, the patient is given an appointment to attend clinic on either the same day or the following day. Exacerbating patients re-visit the clinic at days 3, 7, 14, 35 and 56 post-exacerbation presentation in order to monitor recovery.

1.3.2. COPD and micro-organisms

Bacterial, viral and fungal infections cause a range of upper and lower respiratory tract illnesses in the general population. In healthy individuals, such infections are usually swiftly resolved following diagnosis. COPD drives an inflammatory response in the airways which results in an impaired immune response. This is coupled with the fact that as many as 70% of COPD patients take anti-inflammatory corticosteroids (Zervas et al. 2013), further altering the immune profile of the lungs. It is with this background that the role of micro-organisms should be considered with relation to COPD.

1.3.3. *H. influenzae* virulence mechanisms

The species was initially identified in the late 19th century by the German scientist Richard Pfeiffer, and was classified as *Bacillus influenzae*, due to its association with an influenza epidemic. It was ultimately reclassified as *Haemophilus influenzae* in the early 1900s. This name was retained despite the identification of influenza virus as the cause of influenza in 1933. In 1931 Margaret Pittman made the key finding that there were different strains of the species, and that these were differentiated based upon the presence or absence of a capsule (Pittman 1931). Unencapsulated strains are unable to react with antisera and so cannot be typed according to serology. They are therefore referred to as non-typeable *Haemophilus influenzae* (NTHi) (Murphy et al. 2009).

NTHi are considered to be the major group of *H. influenzae* associated with COPD exacerbations. Oral immunisation with inactivated *H. influenzae* has been shown to reduce rates of moderate-to-severe exacerbation in frequent exacerbators by 63% (Tandon et al. 2010). There are a number of factors which have been shown to have a role in *H. influenzae* virulence: The lipopolysaccharide (LPS) of *H. influenzae* acts as an endotoxin and stimulates secretion of pro-inflammatory cytokines by host cells (Matsui et al. 2001). Furthermore, *in vitro* studies have demonstrated that LPS assists in adherence of *H. influenzae* to human bronchial epithelial cells (Swords et al. 2000). Using LPS-coated polystyrene beads, Swords and colleagues showed that beads coated with wild-type LPS adhered significantly more to bronchial epithelial cells than beads coated with a truncated version of the same LPS.

During infection, NTHi initially adheres to epithelial cells of the upper airway (Erwin & Smith 2007). There are a number of processes which NTHi may then undergo: (i) invasion of respiratory epithelium; (ii) transcytosis into sub-epithelial compartment; (iii) microcolony formation. A number of strains of NTHi are able to express pili, which are threadlike appendages capable of adhering to airway epithelial cells (Geme 2002) and the presence of pili has been shown to increase adherence of NTHi to respiratory mucus (Kubiet et al. 2000).

1.3.4. *S. pneumoniae* virulence mechanisms

More than 90 serotypes of *S. pneumoniae* have been identified. The organism has been shown to be present asymptomatically in the nasopharynx of approximately 40% of adults in developing countries (Obaro & Adegbola 2002). Pathogenesis of *S. pneumoniae*, which involves invasion and dissemination of the organism, is most frequently associated with 20 of these serotypes (Hammerschmidt et al. 2005). There are a number of factors linked to causing increased pathogenesis by *S. pneumoniae*. These include:

- A capsular polysaccharide layer
- Phosphorylcholine adhesion
- Pneumococcal surface protein A (PspA)
- Pneumococcal adhesion and virulence factor A (PavA)
- Pneumolysin

The capsular polysaccharide layer (cps) defines more than 90 serotypes of *S. pneumoniae* (Cobey & Lipsitch 2012). The virulence of the cps varies significantly between serotypes, with some serotypes offering better evasion for the organism from complement-mediated opsonisation (Winkelstein 1984). It has also been demonstrated that the cps prevents mucosal entrapment of *S. pneumoniae* (Nelson et al. 2007), which would otherwise prevent transit to the epithelial surface.

Phosphorylcholine (ChoP) is an adhesin common to a number of bacterial species, including *H. influenzae* and *S. pneumoniae*. ChoP binds to the receptor for platelet-activating factor, with this receptor being found in a variety of host tissue, including the nasopharyngeal epithelium (Kadioglu et al. 2008). Via this adherence, ChoP is able to assist invasion of bronchial cells (Swords et al. 2000).

An important branch of the innate immune system is lactoferrin, a protein which is involved in iron sequestration. This prevents bacteria from acquiring iron, thereby restricting growth (Shaper et al. 2004). *S. pneumoniae* expresses a surface protein called Pneumococcal surface protein A (PspA) which binds to apolactoferrin (a form of lactoferrin), conferring protection on the bacterium from such bactericidal properties (Shaper et al. 2004). It has also been demonstrated that PspA is involved in interfering with the host complement response, thereby inhibiting clearance of the bacterium (Tu et al. 1999).

Animal models have demonstrated the virulence potential of the *S. pneumoniae* cell surface molecule, Pneumococcal adhesion and virulence factor A (PavA). In a mouse model of sepsis infection, PavA-null mutants showed more than 100-fold attenuation of virulence (Holmes et al. 2001).

1.3.5. *M. catarrhalis* virulence mechanisms

Moraxella catarrhalis (previously known as *Branhamella catarrhalis*) is a gram-negative diplococcus commonly isolated as a commensal in the upper respiratory

tract of healthy individuals (Murphy & Parameswaran 2009). The rate of carriage is 3-5% in healthy adults (de Vries et al. 2009). The bacterium is now established as a pathogen in both upper and lower respiratory tract infections, including acute exacerbations of COPD (Verduin et al. 2002).

Pathogenesis of *M. catarrhalis* is thought to be dictated by a number of virulence factors, including its lipo-oligosaccharide surface component and various outer membrane molecules (de Vries et al. 2009). These confer a variety of traits upon the bacterium, such as epithelium adherence and iron uptake (Plamondon et al. 2007).

1.3.6. Atypical airway bacteria

Atypical airway bacteria consist primarily of *Chlamydophila pneumoniae*, *Legionella pneumophila*, and *Mycoplasma pneumoniae*. They are known to be common causes of atypical pneumonia, so called as it demonstrates symptoms distinct from those caused in 'typical' pneumonia (Hindiyeh & Carroll 2000). The atypical airway bacteria have been seldom identified in patients with COPD through conventional culture techniques. This may be due to the fact that *C. pneumoniae* is an obligate intracellular pathogen, and both *L. pneumophila* and *M. pneumoniae* have intracellular components to their life cycle. although serological analysis has hinted at a higher prevalence of infection with these species in exacerbating COPD patients compared with controls, as previously described in section 1.1.10. Given the lack of

supporting data from other studies, it would be important to determine the veracity of such findings using molecular techniques.

1.3.7. Bacterial load and strain-switching hypotheses

It is widely accepted that typical airway bacteria trigger acute exacerbations of COPD. However, what is not so clearly defined is the mechanism by which this is caused. One theory suggests that bacterial load is the major driver as it is strongly associated with inflammatory markers (Hill et al. 2000), whilst another hypothesis suggests that intra-species switching of bacterial strains is the trigger for exacerbations (Sethi et al. 2002). The current thesis explores changes in bacterial load in patients at various disease states, and their relation to clinical outcomes, in Chapters 4, 5 and 6.

1.3.8. Airway microbiome

Historically, the healthy lungs have not been thought to be associated with any form of microbiota, and indeed have been believed to be sterile, with any inhaled bacteria thought to have been rapidly phagocytosed by alveolar macrophages. However, recent developments in the field have proved that a bacterial flora – collectively referred to as a microbiome - does exist in the lungs (Hilty et al. 2010; Erb-Downward et al. 2011; Huang et al. 2010).

The presence of an airway microbiome poses a number of questions with respect to the impact of bacterial infection in COPD. In particular, what is the impact of

antibiotic therapy to this microbiome, and does the microbiome have a pathogenic effect in acute exacerbations of COPD?

The respiratory tract is a continuous tract running from the buccal orifice down to the lung alveoli. It is conventionally sub-divided into an upper respiratory tract (URT) and lower respiratory tract (LRT). The URT consists of the nasal cavity, pharynx and larynx, whilst the LRT is made up of the trachea, bronchi, bronchioles and lung alveoli. This sub-division is an important facet of bacterial and viral infection in the respiratory tract, particularly with regards to disease severity – LRT infection is typically associated with more severe symptoms compared with URT infection. The current study investigates changes in the airway microbiome at different states of COPD in chapter 6 and chapter 7.

1.3.9. Detection of bacterial infection

Detection of bacterial infection has been conventionally performed using routine microbiological culture. However with the advent of nucleic acid amplification tests (NAATs) such as PCR, there has been a move towards molecular protocols. A study involving 30 COPD patients has previously been performed to examine the proficiency of PCR and routine microbiological culture in detecting typical airway bacteria (Curran et al. 2007). This indicated that PCR was a more accurate technique for bacterial detection. As part of the validation process of the techniques performed in the present thesis, this comparison is performed using a multiplex PCR in Chapter 3.

The prevalence of typical and atypical airway bacteria in COPD has hitherto been little studied. The present thesis examines the prevalence and quantity (load) of such bacteria at various phases of COPD in Chapters 4, 5, 6 and 8.

1.3.10. Viral infection

Unlike bacteria, viruses are not thought to colonise the airways on a long-term basis. However, viruses have frequently been identified in patients suffering AE-COPD, with rhinovirus particularly prevalent. In an experimental model, Johnston and colleagues found that rhinovirus infection was able to induce exacerbations in stable COPD patients (Mallia et al. 2011). This provides direct evidence of rhinovirus as a causative factor in COPD exacerbations. There is also evidence that patients with persistent RSV infection have higher airway inflammation and faster FEV_1 decline than patients without persistent RSV infection (Wilkinson et al. 2006a).

1.3.11. Bacterial and viral coinfection

It has been demonstrated that exacerbations exhibiting simultaneous bacterial and viral coinfection have higher levels of inflammatory markers (Wilkinson et al. 2006b). Separately, it was identified that rhinoviral infection predisposed COPD patients to opportunistic bacterial infection in 60% of patients, post-rhinoviral infection (Mallia et al. 2012). Those patients with secondary bacterial infection had significantly raised levels of neutrophil elastase – this was not identified in those patients who had no bacterial infection. This may lead to bacterial complications of

viral-induced exacerbations, potentially amplifying an exacerbation (Molyneaux et al. 2013).

1.3.12. Comorbidities

It has become apparent that COPD is associated with disease manifestations other than just airflow limitation (Carlin 2012). In fact there are a number of systemic manifestations of COPD, including cardiovascular diseases such as ischaemic heart disease (Patel et al. 2012), heart failure, osteoporosis, depression and anxiety (Barnes & Celli 2009), and gastro-oesophageal reflux disease (Casanova et al. 2004). As such, COPD patients with comorbidities are at increased risk of hospitalisation, resulting in greater healthcare costs, as well as increased mortality (Barnes & Celli 2009).

1.3.13. Bacterial infection in other airway infections

Bacterial infection is associated with a number of lung diseases distinct from COPD, and an understanding of the impact of such infection may contribute to the knowledge of the role of bacteria in COPD. Cystic fibrosis (CF) is a hereditary condition affecting approximately 1 in 2500 individuals in a Caucasian population (Raman et al. 2002). Bacterial infection is associated with a worsening of CF. For example, *Pseudomonas aeruginosa* infection involves the formation of a biofilm within the lung. This biofilm is protective against both the host immune system and antibiotic treatment (Bjarnsholt et al. 2009). NTHi is known to frequently infect CF patients, and is also associated with the formation of biofilms on the airway

epithelium (Starner et al. 2006). Non-CF bronchiectasis involves a widening of the bronchi following degradation of muscle and elastic tissue. This is triggered by inflammatory/infectious damage, and results in chronic cough and sputum overproduction (O'Donnell 2008). Bacterial infection is commonly associated with bronchiectasis, with the major pathogens isolated being *H. influenzae*, *P. aeruginosa*, *S. pneumoniae*, and *M. catarrhalis*. Patients with *P. aeruginosa* demonstrate an accelerated decline in lung function (Martínez-García et al. 2007), whilst conversely, patients with no pathogen detected have the mildest disease (King et al. 2007).

1.4. Questions to be answered in bacteriology of COPD

Over the last half-century, since the British and Dutch hypotheses were proposed, there has been a significant wealth of information concerning the progression of COPD, not least of which involves the role that infection plays in the development and duration of acute exacerbations of the disease. We now know that typical airway bacteria are associated with exacerbations. However, it is still under considerable debate as to how this association may be manifesting changes in COPD patients. It is of importance to understand the different nature of bacteriology in the stable state, at exacerbation, and during exacerbation recovery, as this shall enhance our knowledge of the changes occurring in individual patients, and provide insights as to how they may be best treated.

1.5. Aims and objectives

The overall aim of this thesis is to investigate the role of bacterial infection in the natural history of chronic obstructive pulmonary disease. This will be assessed in a number of different methods:

- Initial comparison of conventional microbiological culture *versus* a real-time multiplex PCR methodology in order to determine which method is most suitable to detect presence of typical airway bacteria (**Chapter 3**)

- Prevalence and load of typical airway bacterial species suspected of involvement in acute exacerbations of COPD will be measured, at various disease states, in patients from the London COPD Cohort, using the appropriately determined method (**Chapter 4**)

- Prevalence and load of atypical airway bacterial species will be examined using a real-time multiplex PCR approach, at stable and exacerbation state (**Chapter 8**)

- Overall load of the airway microbiome of London COPD Cohort patients will be assessed at different states of COPD using a modified version of a previously-validated 16S real-time PCR method (**Chapter 6, 7**)

- Determine whether changes occur in airway bacterial prevalence and load in the recovery period following an exacerbation (**Chapter 6**)

- Examine whether presence of airway bacteria during stable COPD is related to clinical parameters such as lung function (**Chapter 5**)

- Assess relationship between airway bacterial load with inhaled corticosteroid dosage in order to follow up on previous literature suggesting an association between ICS use and pneumonia (**Chapter 5**)

- The rate of presence of atypical airway bacteria will be assessed in London COPD Cohort patients at both the stable and exacerbation phases (**Chapter 7**)

- Is presence of airway bacteria associated with systemic inflammation in COPD patients? (**Chapter 4, 5, 6, and 7**)

This will be the first study in which the relationship between typical airway bacteria, atypical airway bacteria and the total lung microbiome is characterised within the same group of patients. This data collection allows for a significant amount of follow-up analyses:

- Is inflammation driven by any bacterial species in particular? (**Chapter 4**)

- Does the airway microbiome persist during and after antibiotic therapy following an acute exacerbation of COPD? (**Chapter 6**)

- To what extent does typical airway bacterial prevalence and load change between exacerbation presentation and the recovery period? (**Chapter 6**)

- Does persistence of typical bacteria during exacerbation recovery increase the risk of recurrent exacerbations? (**Chapter 6**)

- Is the overall load of the microbiome affected by presence or absence of typical airway bacteria? (**Chapter 6, 7**)

- Does airway microbiome load correlate to typical airway bacterial load? (**Chapter 6**)

Ultimately, the answers to these questions will provide significant contributions to our understanding of the role of typical and atypical airway bacterial infection in exacerbations, and will also help us to understand the changes that occur to the commensal airway microbiome in the face of such an insult.

CHAPTER 2. **Materials and Methods**

2.1 London COPD cohort – funding and ethics

The London COPD cohort is a rolling cohort of approximately 200 patients enrolled as part of a long-term study at the Centre for Respiratory Medicine, Division of Medicine, University College London. This is an observational study exploring the relationship between patient phenotypes and exacerbations in COPD.

The London COPD cohort is funded by the Medical Research Council, United Kingdom (Ref. G0800570).

Ethical approval was granted by the Royal Free London National Health Service Foundation Trust (Ref. 09/H0720/8), and informed written consent was obtained from the patients, allowing sputum and blood samples to be collected and spirometry readings to be taken. Approval was also given to allow daily diary card data to be obtained from patients (explained further in section 2.3.2).

2.2 Recruitment

Patient recruitment was performed either through referral (e.g. by General Practitioner) or when a patient was seen by a London COPD cohort clinician, whereby the Cohort Study was explained to the subject, who was then able to opt in or out of the study (Seemungal et al. 1998). Patients were allowed to leave the study at any point. To ensure confidentiality, each subject was assigned with a unique study number. Patients were included if the forced expiratory volume in one second (FEV_1) was ≤80% predicted and FEV_1/forced vital capacity (FVC) ratio was <0.7, in keeping with GOLD stages II-IV (Rabe et al. 2007). A history of chronic symptoms (dyspnoea, sputum production, wheeze and cough) was taken, as well as smoking history (number of pack-years smoked, current smoking status). Patients with a history of any other significant respiratory diseases were excluded, as were those unable to complete daily diary cards.

At recruitment, the following patient characteristics were collected and recorded by the attending clinician (a clinician of the London COPD Cohort): age, gender, height, smoking status, pack-year history, maintenance therapy details, and spirometry details (FEV_1, FVC). Spirometry measurements were obtained using a volumetric storage spirometer (Vitalograph 2160; Maids Moreton, Buckingham, UK). All recruited patients had an FEV_1 <80%, predicted from age, gender and height, and an FEV_1/FVC ratio <0.7. Patients with significant non-COPD respiratory disease were excluded.

2.3 Subjects

2.3.1 Patient visits

Patients visited the clinic as outpatients in one of a number of different states. They were seen either at stable state, exacerbation state, or during recovery time points post-exacerbation (days 3, 7, 14 and 35 post-exacerbation). An exacerbation was defined as new or increased respiratory symptoms for two or more consecutive days, with at least one major symptom (dyspnoea, sputum purulence and sputum volume), and another major or a minor symptom (wheeze, cold, sore throat and cough), the first day of which was defined as the day of onset of the exacerbation. Stable state was defined as no symptom-defined exacerbations for the preceding 4 weeks and the subsequent 2 weeks post-clinic visit. At each visit, spirometry data was recorded, as well as any changes in maintenance therapy. Diary card data was also recorded by the patients on a daily basis (see section 2.3.2 and appendix). Patients were also given access to a 24-hour hotline with direct access to a Respiratory physician, to discuss any concerns they may have had with their illness.

2.3.2 Community-based data acquisition with daily diary cards

Daily diary cards recorded a number of different parameters. Peak expiratory flow rate, which quantifies the patient's maximal expiratory flow (in litres/minute) was self-measured using a volumetric storage spirometer. The presence of or increase of minor/major respiratory symptoms compared with the stable state was noted. This allowed for the identification of exacerbations which were not reported to the COPD clinic – known as 'unreported exacerbations'.

2.4 Sputum sampling

Patients produced sputum either spontaneously, or through sputum induction. Sputum induction was administered by either the Respiratory physician or Respiratory nurse. Nebulisation was carried out with 3% saline, using a protocol modified from the procedure described by Pin and colleagues (Pin et al. 1992). This modified protocol has been previously described by our group (Bhowmik et al. 1998). Nebulisation was performed using the DeVilbiss UltraNeb2000 ultrasonic nebuliser (DeVilbiss healthcare, Tipton, UK). This nebuliser produces an aerosol output of 2ml/minute, with a mean particle size of between 0.5-5µm in diameter. In brief, the process involved seven minutes of nebulisation, after which oxygen saturation and spirometric tests were performed, prior to sputum expectoration. As long as the FEV_1 had not fallen by more than 20%, nebulisation continued for an additional seven minutes, after which further expectoration occurred.

2.4.1 Sputum processing

Sputum was processed after being obtained from the patient, and was performed by either the Respiratory physician or the laboratory technician (Ray Sapsford).

Initially the sputum sample was weighed and diluted ten-fold with phosphate-buffered saline (PBS) (Sigma; P-4417) e.g. if the sputum sample weighed 0.5g, it would be diluted in 4.5ml PBS. If the physician required, any excess sputum was sent to the clinical microbiology department for microbiological culture analysis.

Sterile glass beads (2.5-3.5mm; 33212 4G, VWR) were then added to the sputum-PBS suspension, and the mixture was homogenised by being vortexed (Whirlimixer, Fisons, Ipswich, UK) for 15 seconds, followed by shaking on an IKA VIBRAX tube shaker (IKA Werke GmbH & Co.KG, Germany). The homogenised sample was then aliquoted as 500µl volumes into microcentrifuge tubes, and kept in long-term storage at -80^{o}C. This sample was then able to be processed for DNA extraction at a later date.

2.5 DNA extraction from sputum samples

The homogenised sputum sample was retrieved from the -80^{o}C freezer and thawed at room temperature for 20-25 minutes. Once thawing was completed, the samples underwent heat-killing at 90^{o}C in a heat block (Dri-block, Techne, Staffordshire, UK) for 30 minutes. The 500µl sample was then transferred to a fresh UV-sterilised 1.5ml microcentrifuge tube. Samples were spun in a microfuge (Eppendorf, 5415C) at 13000g for 10 minutes. Supernatant was carefully decanted and the pellet was resuspended in 1ml of PBS (at a 1x working concentration), in a washing step.

This suspension then underwent vortexing at maximum speed for 10 seconds to allow the pellet to mix with the PBS. Samples were spun in a microfuge at 13000g for 10 minutes. The supernatant was carefully decanted and the wash step repeated. Following removal of the supernatant, the washed pellet was resuspended in 200µl of PCR-grade UV-sterilised water (Sigma-W4502). The sample underwent vortexing at maximum speed for 10 seconds to allow thorough mixing to occur. A volume of 200µl of 10% Chelex 100 (Sigma C-7901) was added to each sample – note that this required use of a 1000µl pipette tip as 200µl tips are not wide enough to easily take up 10% Chelex 100.

The sample was then thoroughly vortexed for 20 seconds at maximum speed to ensure that the Chelex adequately mixed in with the sample. It was then transferred to a heat block pre-heated at 56^{o}C, where it was incubated for 20

minutes – this allowed Chelex to bind metal cofactors such as Mg^{2+} ions whilst limiting the activity of nucleases.

Following incubation, the sample was vortexed for 10 seconds at maximum speed and then heated at 95^{o}C for 5 minutes, in order to ensure denaturation of nucleases. The sample was then cooled on (wet) ice prior to centrifugation at 16000g for 10 minutes. The supernatant, containing the extracted DNA, was transferred to a fresh UV-sterilised 1.5ml microcentrifuge tube and stored at 4^{o}C.

2.6 PCR for detection and quantification of bacteria

In total, 3 different PCR protocols were utilised: (i) a multiplex PCR for the detection and quantification of the typical airway bacteria *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* (Abdeldaim et al. 2008; Greiner et al. 2003; Garcha et al. 2012); (ii) a multiplex PCR for the detection and quantification of the atypical airway bacteria *C. pneumoniae*, *L. pneumophila* and *M. pneumoniae* (Ling & McHugh 2013); (iii) a PCR targeting a highly conserved region of the gene encoding 16S ribosomal RNA, for the detection and quantification of all bacterial DNA (Erb-Downward et al. 2011).

Each PCR protocol employed hydrolysis probes (TaqMan probes; Tib MolBiol) in order to quantify the amount of bacterial DNA present in a sample (Table 2.1, Table 2.7, Table 2.8). As part of the current study, it was desired that the amount of bacterial DNA measured for each species be converted into the units of colony forming units (cfu)/ml, in order to more appropriately relate it to previous literature. This was performed for the atypical and typical airway bacterial PCRs (with the exception of *C. pneumoniae*, for which copies/ml would be reported, due to fastidious growth conditions).

	Sequence (written 5' - 3')
Typical primers	
Spn-F	AgTCgTTCCAAggTAACAAgTCT
Spn-R	ACCAACTCgACCACCTCTTT
Hin-F	CCgggTgCggTAgAATTTAATAA
Hin-R	CTgATTTTTCAgTgCTgTCTTTgC
Mor-F	gTgAgTgCCgCTTTTACAACC
Mor-R	TgTATCgCCTgCCAAgACAA
SPUD-F	AACTTggCTTTAATggACCTCCA
SPUD-R	ACATTCATCCTTACATggCACCA
Typical probes	
Spn-TM	ROX-TACATgTAggAAACTATTTTCCTCACAAA--BHQ2
Hin-TM	6FAM-ACAgCCACAACggTAAAgTgTTCTACg--DB
Mor-TM	6JOE-TgCTTTTgCAgCTgTTAgCCAgCCTAA--BHQ1
SPUD-P	Cy5-TgCACAAgCTATggAACACCACgT--BBQ

Table 2.1. List of primers and probes used for typical airway bacterial PCRs. Typical primers/probes purchased from TIB MOLBIOL.

For the 16S gene PCR, the reported unit was copies/ml, as different bacterial species have varying copies of the 16S gene. In each case, formation of relevant standard curves using positive controls was required.

2.6.1 Correlation of colony-forming units with DNA concentration

For the typical airway bacteria, as well as *L. pneumophila* and *M. pneumoniae*, ATCC strains of these species were subcultured onto relevant media and incubated to obtain pure plates.

A selection of 10 colonies were picked up with a sterile plastic loop and inoculated into microcentrifuge tubes containing 600µl of PCR-grade H_2O. Of this suspension, 500µl was to be used to create a DNA extract. This DNA extract was established using the same method as outlined in section 2.5. The DNA concentration was then determined using a Nanodrop spectrophotometer (Thermo Scientific, Delaware, USA). The remaining 100µl of the suspension underwent serial dilution, as described in section 2.6.1.1.

2.6.1.1 Quantification of colony forming units

For each species, ten-fold serial dilutions were created from neat down to 10^{-8}. Each of the serial dilutions were then plated as 20µl spots and incubated at $35^{o}C$ with 5% CO_2. After 48 hours of incubation, bacterial colony counting was performed, using the Miles & Misra method (Miles et al. 1938).

2.6.2 Standard curve preparation to quantify PCR amplimers

For the typical airway bacteria, as well as *L. pneumophila* and *M. pneumoniae*, standard curves for the quantification of PCR amplimers were prepared using the serial dilutions. These dilutions for each species underwent DNA extraction (see section 2.5), and 5µL of DNA extract underwent PCR amplification. For *C. pneumoniae*, due to difficulty in performing routine microbiological culture, copies of the genome were purchased and used as the basis for the curve, following serial dilution.

For the 16S PCR, a single species was used as the template for the standard curve (*H. influenzae*). Extracted *H. influenzae* DNA was serially diluted down to 10^{-8} (in order to reduce genomic DNA content to insignificant amounts) and this 10^{-8} dilution underwent conventional PCR in order to amplify the 16S target gene. Mastermix details are shown in Table 2.2, with 5µl of extracted DNA added to this mastermix. Primer details for the 16S gene are located in Table 2.8. Five microlitres of diluted *H. influenzae* was used as template DNA. The following PCR conditions were utilised: 50^{o}C for 2 minutes, 95^{o}C for 10 minutes, 45 x (95^{o}C for 15 seconds, 60^{o}C for 60 seconds).

Positive controls were created by spiking negative sputum samples with ATCC strains of bacterial species, which underwent DNA extraction as described in Section 2.5.

Reagents	Volume (µl)
Platinum qPCR supermix	12.5
16S forward primer (10µM)	0.5
16S reverse primer (10µM)	0.5
H_2O	6.5

Table 2.2. Mastermix for conventional 16S PCR amplification. Platinum qPCR supermix obtained from Invitrogen (Cat. No. 11730-025). H_2O obtained from Sigma (W4502)

Following amplification, the PCR product underwent 1.5% agarose gel electrophoresis in 1x TBE buffer, to confirm presence of a band of the size expected from the 16S amplicon. PCR purification (QIAquick PCR purification kit, Qiagen) was performed to clean up the PCR product for downstream real-time PCR analysis: 5x volume of buffer PB was added to 1x volume of PCR product. The sample was then applied to a QIAquick spin column and centrifuged at 17900g for 60 seconds – flow through was discarded. The DNA was then washed by addition of 0.75ml buffer PE and centrifugation at 17900g for 60 seconds. This step was repeated to ensure removal of residual ethanol. The QIAquick spin column was then placed into a fresh 1.5ml microcentrifuge tube. Elution of DNA was carried out by adding 30µl of buffer EB to the QIAquick membrane in the column, leaving to stand for 60 seconds, and then centrifuging at 17900g for 60 seconds.

2.7 Quantitative PCR for typical airway bacteria

Quantitative PCR (qPCR) was performed on *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* using a multiplex PCR developed at the Centre for Clinical Microbiology, University College London (Garcha et al. 2012). This was performed on either a Rotor-gene 3000 or Rotor-gene 6000 real-time PCR (Corbett Research UK, Cambridgeshire, UK).

The gene targets for the three bacterial species are outlined in Table 2.3. The targets for *M. catarrhalis* (Greiner et al. 2003) and *S. pneumoniae* (Abdeldaim et al. 2008), had previously been validated as appropriate targets for the relevant species. The *H. influenzae* gene target (Hel) was validated in-house at the UCL Centre for Clinical Microbiology. This validation was performed by Dr Clare Ling and Julianne Lockwood.

Species	Gene target	Reference
H. influenzae	Hel gene (encodes P4 lipoprotein)	In-house
M. catarrhalis	CopB (encodes outer membrane protein)	Greiner et al (2003)
S. pneumoniae	spn9802 (gene fragment)	Abdeldaim et al (2008)

Table 2.3. Target genes for typical airway bacteria PCR.

An internal amplification control (IAC) was utilised in this PCR in order to detect any PCR inhibition, incorporated in the typical airway PCR mastermix (Table 2.4). The IAC used was known as SPUD - this targeted the *PhyB* gene from the potato species *Solanum tuberosum*, as this had been previously validated for use as an IAC (Nolan et al. 2006). Primer/probe details for SPUD are described in Table 2.1.

Reagents	Volume (µl)
Platinum qPCR Supermix	12.5
$MgCl_2$ (50mM)	1.5
Forward primers for bacterial DNA (50µM)	0.075
Reverse primers for bacterial DNA (50µM)	0.125
IAC forward primer (50µM)	0.125
IAC reverse primer (50µM)	0.125
Probes for bacterial DNA (50µM)	0.125
IAC probe (50µM)	0.1
IAC	1
H_2O	3.675

Table 2.4 Reagents used to make up mastermix for multiplex typical airway bacteria qPCR. This represents the volume required per PCR reaction. IAC = internal amplification control.

As the IAC was placed directly into the PCR mastermix, each PCR tube contained equal quantities of this gene. Therefore any significant fluctuation in detected concentration following the PCR would be due to inhibition, and could be corrected for.

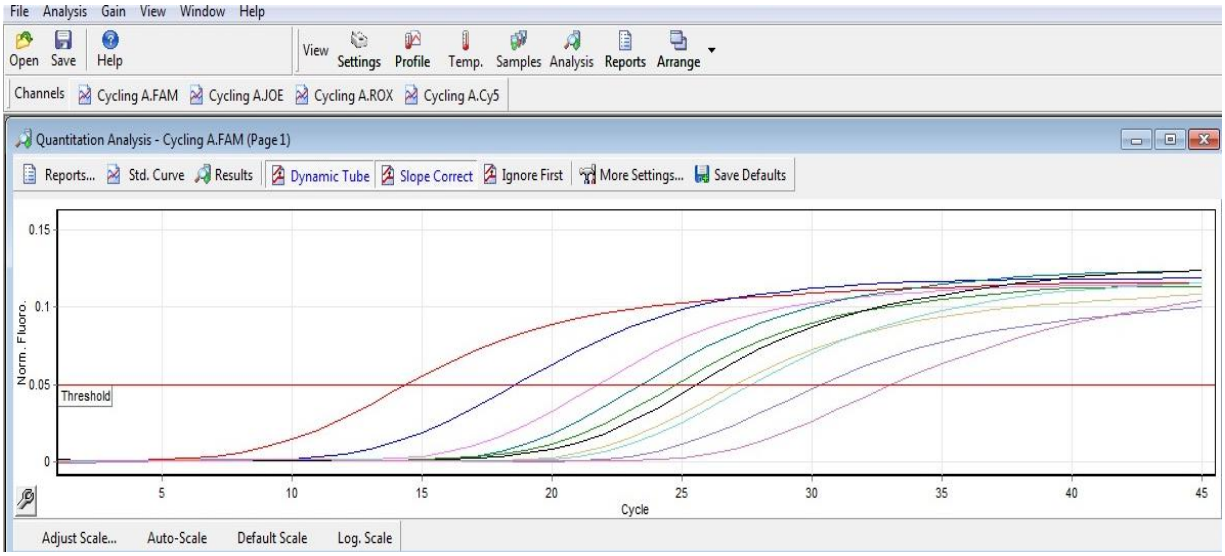
2.7.1 Typical airway bacteria PCR protocol

The mastermix illustrated in Table 2.4 formed the basis of each typical airway bacterial PCR reaction. Twenty microlitres of mastermix was added to each tube. Samples were added to tubes in duplicate, at a volume of 5µl per tube, creating a total reaction volume of 25µl.

As the standard curves for each species had already been created (as described in section 2.6), a single standard from this curve was required for use in each run, acting as a positive control, ensuring that the PCR reaction ran successfully. It also allowed load quantification to be determined for any positive samples. Cycling conditions for the typical airway bacterial PCR were as follows: 95^{o}C for 3 minutes; 45 cycles of (95^{o}C for 10 seconds, 60^{o}C for 45 seconds). The primer and probe sequences are described in Table 2.1.

Standard curves were then developed by running PCRs with dilutions of the individual species (Figure 2.1).

A



B

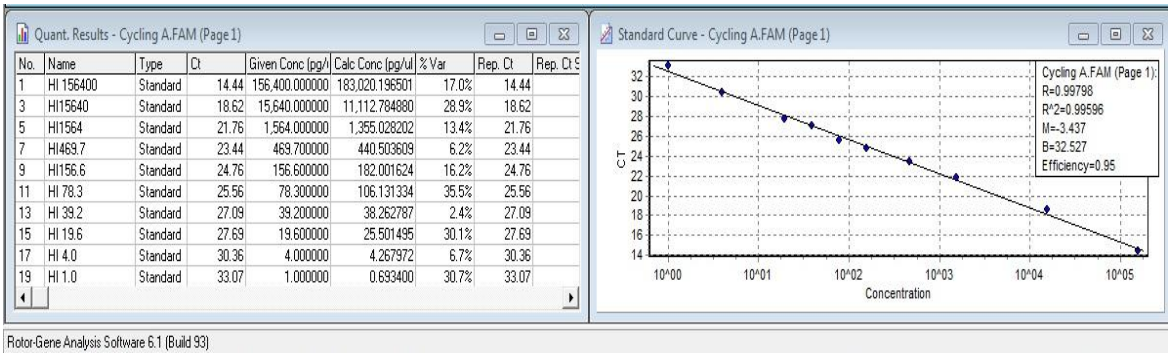


Figure 2.1. Standard curve generation for absolute quantification of bacterial load. (A) Serial dilutions of known quantities of DNA ran on PCR. In the example in this figure, the dilutions of *H. influenzae* are illustrated. The concentration ranged from 156400 pg/µl, down to 0.5 pg/µl (the lowest concentration to show positivity was 1 pg/µl) (B) Standard curve generated, relating DNA concentration to Ct value.

2.8 Quantitative PCR for atypical airway bacteria

A quantitative multiplex PCR was established to detect the atypical airway bacteria *C. pneumoniae*, *L. pneumophila* and *M. pneumoniae*. This PCR was developed at the Centre for Clinical Microbiology, University College London (Ling & McHugh 2013). This was performed on a Rotor-gene 6000 real-time PCR (Corbett Research UK, Cambridgeshire, UK). The target genes are listed in Table 2.5, with primer and probe details described in Table 2.7.

Species	Gene target
C. pneumoniae	RNA polymerase β-chain gene
L. pneumophila	*mip* gene
M. pneumoniae	P1 adhesin protein gene

Table 2.5. Target genes for typical airway bacteria PCR.

Instead of using an IAC, the atypical PCR employed two mastermixes in order to detect and correct for any inhibition (Table 2.6). This second mastermix contained 1µl of a known amount of *L. pneumophila* DNA (5ng/µl). This was substituted in for 1µl of DNA extract. For each DNA extract sample, both mastermixes were utilised.

Any concentration decrease seen in the cycle threshold value of *L. pneumophila* in the PCR results of the second mastermix would therefore be an indicator of inhibition. Cycle conditions were as follows: 95°C for 3 minutes, 40 x (95°C for 12 seconds, 60°C for 45 seconds).

Reagent	Spiked MM (1X)	Unspiked MM (1X)
Platinum Supermix	12.5	12.5
MgCl2 (50mM)	1.5	1.5
Forward primers (10µM)	0.75	0.75
Reverse primers (35µM)	0.357	0.357
Probe (10µM)	0.625	0.625
Water	0.803	0.803
Legionella DNA (5ng/µl)	1	N/A

Table 2.6. Reagents used to make up mastermix for multiplex atypical airway bacteria qPCR. This represents the volume required per PCR reaction. MM = Mastermix

2.8.1 Atypical airway bacteria PCR protocol

The mastermixes described in Table 2.6 were used as the basis for the PCR. Each DNA extract sample was run in duplicate with the unspiked mastermix, and singly with the spiked mastermix. Five µl of sample was added to each unspiked mastermix, whilst 4µl of sample was added to each spiked mastermix. Therefore each reaction had a total volume of 25µl. Primer and probe sequence details for the atypical airway bacteria PCR are listed in Table 2.7.

	Sequence (written 5' - 3')
Atypical primers	
Lp-F	AAAGGCATGCAAGACGCTAT
Lp-R	GTACGYTTTGCCATCAAATCTT
Mp-F	GGTCAACACATCAACCTTTTGGT
Mp-R	TGTGATTGTGCTCAGTGTTACCT
Cp-F	CATGGTGTCATTCGCCAAGT
Cp-R	CGTGTCGTCCAGCCATTTTA
Atypical probes	
Lp-probe	ROX-TGTTAAGAACGTCTTTCATTTGCTGTTCGG-BHQ2
Mp-probe	YAK-ACCCAGCCTTCAAGGCCTGTTTGTTCTTGT-DB
Cp-probe	6FAM-TCTACGTTGCCTCTAAGAGAAAACTTCAAGTTGGA-DB

Table 2.7. List of primers and probes used for atypical airway bacterial PCR. Primers/probes purchased from TIB MOLBIOL.

2.9 Quantitative PCR for 16S ribosomal DNA

Quantitative PCR was performed, targeting a highly conserved region of the gene encoding 16S ribosomal RNA. This region is ubiquitous amongst bacterial species, and so allowed for total bacterial load in a sample to be determined. The protocol employed was a modified version of a previously-described procedure (Erb-Downward et al. 2011). The modification involved incorporation of an internal amplification control (SPUD). Optimisation for this modified protocol was performed by myself and Laura Wright (Centre for Clinical Microbiology, University College London).

2.9.1 16S quantitative PCR optimisation

The primers and probes utilised in the PCR are described in Table 2.8.

	Sequence (written 5' - 3')
primers	
16S-F	TCCTACGGGAGGCAGCAGT
16S-R	GGACTACCAGGGTATCTAATCTT
probe	
16S-probe	6FAM-CGTATTACCGCGGCTGCTGGCAC-TAM

Table 2.8. List of primers and probes used for 16S airway bacterial PCR. Primers/probes purchased from TIB MOLBIOL.

The initial step towards optimisation involved confirming that the primers would be able to detect a wide range of bacterial species (Table 2.9).

Species (ATCC No.)	Gram type	Shape
Escherichia coli (700928)	Gram-negative	Rod
Streptococcus pyogenes (700294)	Gram-negative	Coccus
Enterococcus faecalis (19433)	Gram-positive	Rod
Pseudomonas aeruginosa (39324)	Gram-negative	Coccobacillus
Enterobacter cloacae (39978)	Gram-negative	Rod
Staphylococcus aureus (700699)	Gram-positive	Coccus
Klebsiella pneumoniae (31488)	Gram-negative	Rod
Haemophilus influenzae (10211)	Gram-negative	Coccobacillus
Streptococcus pneumoniae (49619)	Gram-positive	Diplococci

Table 2.9. Species tested to establish efficacy of 16S quantitative PCR. A diverse range of bacteria were chosen for testing.

ATCC strains of these species were grown overnight in brain heart infusion broth in a shaking 37^{o}C incubator with 5% CO_2. Ten-fold serial dilutions of these cultures were then performed, from neat down to 10^{-7}. Colony counting was then performed as described previously (section 2.6.1.1). Simultaneously, 500µl of each broth underwent Chelex DNA extraction, as previously outlined (section 2.5).

In a method identical to that previously discussed (section 2.6.1), cfu/ml was calculated for each species with the counts shown in Table 2.10 (N.B. *H. influenzae* and *S. pneumoniae* had already undergone this count, and this is recorded in Table 3.3).

Species	cfu/ml in neat sample
Escherichia coli	1.93E+09
Streptococcus pyogenes	5.20E+08
Enterococcus faecalis	5.70E+08
Pseudomonas aeruginosa	1.60E+09
Enterobacter cloacae	3.10E+09
Staphylococcus aureus	1.67E+09
Klebsiella pneumoniae	3.67E+09

Table 2.10. Colony counts for bacterial species used as test organisms to verify efficacy of 16S bacterial PCR

2.9.1.1 DNA extraction and 16S PCR

DNA extraction for the test organism was performed alongside colony counting, as previously described in section 2.5. For the PCR, a mastermix was prepared (Table

2.11). The cycling conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 45 x (95°C for 15 seconds, 60°C for 60 seconds).

Reagents	Volume (µl)
Platinum supermix	12.5
16S forward primer (10µM)	0.5
16S reverse primer (10µM)	0.5
IAC forward primer (50µM)	0.125
IAC reverse primer (50µM)	0.125
16S probe (10µM)	0.625
IAC	1
H_2O	4.625

Table 2.11. Reagents used to make up mastermix for 16S bacterial qPCR. This represents the volume required per PCR reaction. Primers and probes were obtained from TIB MOLBIOL.

In order to analyse whether the 16S PCR would detect all species, test runs were performed examining the DNA extracts from the test organisms. The 16S rRNA gene was successfully detected in all species. One such test run is illustrated, with serial

dilutions of *Pseudomonas aeruginosa*, *Enterococcus cloacae* and *Staphylococcus aureus* (Figure 2.2).

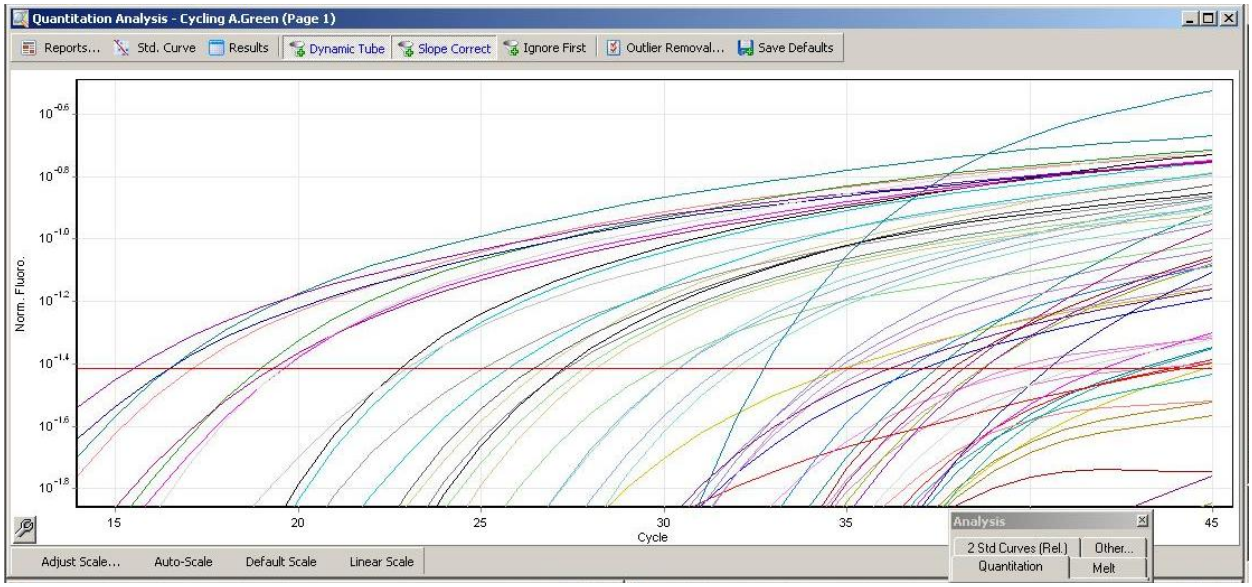


Figure 2.2. Test run examining detection of the 16S rRNA gene in bacterial species. Negative controls were not detected.

It was noted that there was little inter-species correlation with regards to cycle threshold value and bacterial load. For example, for *Pseudomonas aeruginosa*, 10^9cfu/ml was detected at a cycle threshold value of ~16, whilst for *S. aureus*, 10^9 cfu/ml was detected at a cycle threshold value of ~24. This may partly be due to variations in the number of copies of the 16S rRNA gene in genomes of different species. Overall, this indicated that cfu/ml would not be suitable as the unit of measurement of total airway bacterial load, as consistency amongst samples could not be verified. It was therefore determined that a more appropriate measure would be to use the units of copies/ml.

In order to calculate copies/ml, a single species, *H. influenzae*, was used as the standard. Extracted *H. influenzae* DNA was amplified in a conventional PCR. Identical cycling conditions to those used for the 16S PCR were utilised, with the following modifications: no probes were used in the mastermix, with additional H_2O added to make up the volume to 20µl, prior to the addition of 5µl *H. influenzae* DNA. Amplification of DNA was confirmed via gel electrophoresis.

Having confirmed that DNA amplification was successful, the samples underwent PCR purification as described in section 2.6.2.

2.10 Statistical analysis

Data were analysed using SPSS Statistics V.21 (IBM Corp.). The Kolmogorov–Smirnov test for normality was applied. Clinical data with normal distribution are described in mean (±SD). Differences between groups were analysed by independent-samples t test, paired-samples t test, Mann–Whitney U test, one-way ANOVA or Kruskal-Wallis, dependent upon the sample population being investigated. Relationships between continuous variables were analysed using Pearson's or Spearman's correlation in a univariate analysis, or by linear regression in a multivariate analysis. Frequency distribution was explored by χ^2 analysis. A probability of $p<0.05$ was considered to be statistically significant.

CHAPTER 3. **Comparison of Quantitative PCR and Routine Microbiological Culture as Techniques to Detect Typical Airway Bacteria Presence in COPD**

3.1 Introduction

The typical airway bacteria represent the most common causes of bacterial lower respiratory tract infections identified, both in the general population and in COPD. In order to effectively treat patients with a LRTI, rapid and accurate pathogen identification is imperative. This has conventionally been performed using microbiological culture, with culture still in common use today.

However, the advent of sensitive, specific and rapid molecular techniques indicates that well-designed comparisons are warranted to establish whether culture is still the most suitable method of bacterial identification of COPD. These molecular techniques all involve nucleic acid amplification with different advantages and disadvantages, as summarised in Table 3.1.

Nucleic acid amplification technique	Process	Advantages	Disadvantages	Reference
Conventional PCR	Amplification of target DNA sequence	Highly specific, Highly sensitive	Samples may contain PCR inhibitors, skewing results	(Mullis et al. 1986)
Real-time PCR (Quantitative PCR)	Amplification of target DNA sequence with additional use of probe	Increased specificity over conventional PCR; multiple targets in a single PCR run	PCR inhibition; Limit to the number of probes which can be detected by thermocycler	(Opel et al. 2010)
Microarray	DNA is amplified before transferring to a surface containing multiple DNA 'spot' sequences, targeting specific genes	Allows rapid identification of a range of organisms	Cross-hybridisation may occur, in which DNA binds non-specifically to DNA spots causing false-positivity	(Chen et al. 2006)
Nested PCR	Amplification of DNA sequence using two primer sets	high specificity	Time consuming	(Drago et al. 2004)
Isothermal amplification	Amplification of DNA at a constant temperature	No requirement for thermocycler; extremely fast	High level of background 'noise' due to non-stringent temperature; inefficient at amplifying long-target sequences	(Chang et al. 2012; Walker 1993)

Table 3.1. Description of nucleic acid amplification techniques.

Of the cycling amplification methods, a frequently used way to both detect and quantify DNA is quantitative real-time PCR (qPCR). This technique detects and amplifies target DNA, such as a suspected bacterial infection. This allows the quantity, or load, of pathogen to be accurately measured. Inhibition in the PCR reaction can be corrected for by use of an internal amplification control (Nolan et al. 2006). In the current study, expectorated sputum was used as the method of sampling. Alternatives for sampling method include protected specimen brush (PSB) sampling and broncho-alveolar lavage (BAL) fluid sampling. However, both of these methods are invasive and so the ease of sampling provided by sputum was the overriding factor in this decision.

One study has previously examined the rate of identification of bacteria using culture and PCR in 30 COPD patients (Curran et al. 2007). However, that study identified presence of *Haemophilus spp.* as opposed to *H. influenzae*. Given that non-typeable *H. influenzae* is frequently associated with COPD exacerbations (Sethi et al. 2002), it is important to specifically identify the rate of detection for this species by culture and PCR. For *S. pneumoniae*, Curran and colleagues also targeted the *ply* gene in their PCR assay – however, this is known to also detect *S. pseudopneumoniae* and *S. mitis* (Abdeldaim et al. 2008). A more specific target would be the *spn9802* gene fragment, which is present only on *S. pneumoniae* and *S. pseudopneumoniae* (Abdeldaim et al. 2008).

3.1.1 Background of typical airway bacteria in respiratory disease

The typical airway bacteria, *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* are the most commonly identified bacterial community-acquired respiratory pathogens, with each being causative agents of a number of LRTIs. These species are frequent causes of pneumonia. *S. pneumoniae* is the most frequent aetiological agent of bacterial pneumonia and such cases are referred to as pneumococcal pneumonia (Mufson & Stanek 1999). Other airway pneumococcal infections (i.e. those infections caused by *S. pneumoniae*) include bronchitis and sinusitis.

Streptococcus pneumoniae is designated in different serotypes, with each serotype being defined by cell surface antigens present on its capsule (Brito et al. 2003). There are more than 90 known serotypes of the bacterium, (Weinberger et al. 2011), exhibiting varying degrees of pathogenicity. The major serotypes associated with pneumonia are 3, 6A, 6B, 9N and 19F (Weinberger et al. 2010).

Haemophilus influenzae is typed according to it capsule, with six types defined (a-f) (Lis & Górny 2012). Those *H. influenzae* strains lacking a capsule are grouped into the classification of non-typeable *Haemophilus influenzae* (NTHi). NTHi is commonly identified in exacerbations of COPD, with previous studies suggesting a rate of between 22-30% (Miravitlles et al. 1999; Rosell et al. 2005).

Moraxella catarrhalis is a gram-negative organism known to cause pneumonia and otitis media, and it is also associated with acute exacerbations of COPD (Murphy & Parameswaran 2009).

3.1.2 Prevention of typical airway bacterial infection

For pneumococcal infection, a polyvalent vaccine (PCV-7) was introduced in the United States in 2000, and into the UK in 2006, as part of the routine childhood immunisation programmes (Tan 2012; Weil-Olivier et al. 2012). This vaccine targeted seven serotypes of *S. pneumoniae* (serotypes 4, 6B, 9V, 14, 18C, 19F and 23F), which were responsible for causing the majority of severe invasive pneumococcal disease (Hausdorff et al. 2005). More recently, a vaccine targeting 13 serotypes of *S.* pneumoniae has been developed. The UK National Institute for Health and Clinical Excellence (NICE) recommends routine pneumococcal vaccination for COPD patients (NICE 2004, p.012).

A vaccine exists for *H. influenzae* type b infection (Hib vaccine), and this has been part of the UK childhood immunisation programme since 1992 (Heath & McVernon 2002). However, no vaccine exists for the prevention of NTHi, which is the most prevalent form of *H. influenzae* infection in COPD.

Despite considerable investigation, no vaccine currently exists for the prevention of *M. catarrhalis* infection (McMichael 2000; Riesbeck et al. 2006).

3.1.3 Typical airway bacteria in COPD

Typical airway bacteria have been frequently isolated in patients with COPD, during both the stable state and at exacerbation, primarily through microbiological culture (Rosell et al. 2005; Papi et al. 2006). A common pattern identified with these species is that, as expected, there is a higher prevalence detected at exacerbation compared with at the stable state. The study by Rosell and colleagues involved a pooled analysis of a range of different studies, in which the samples were obtained PSB sampling. In PSB, bronchoscopy is performed to obtain an uncontaminated lower airway sample, as previously described (Wimberley et al. 1979; Wimberley et al. 1982). However, the studies did not all use the same definitions to define stable state and exacerbation, and this variation is identified below (Table 3.2).

Reference	Stable COPD definition	Exacerbation definition
(Monso et al. 1995)	40 COPD outpatients who had no evidence of change in sputum purulence/volume, or dyspnoea, in the previous 15 days	29 COPD outpatients. Exacerbation state not defined
(Zalacain et al. 1999)	88 COPD outpatients with stable symptoms and had not taken antibiotics in previous 30 days or corticosteroids in previous 3 months. Not admitted to hospital in previous 6 months	N/A
(Papi et al. 2006)	64 COPD outpatients. Stable state obtained 8-10 weeks *post*-exacerbation	64 hospitalised COPD patients. Exacerbation defined as increased dyspnoea, cough or sputum expectoration requiring medical attention
(Soler et al. 2007)	N/A	50 hospitalised COPD patients, suffering marked increase in dyspnoea, cough and/or purulent sputum expectorate
(Monsó et al. 1999)	41 outpatients with chronic bronchitis. Patients had no evidence of change in sputum purulence/volume, or dyspnoea, in the previous 15 days	N/A

Table 3.2. Description of stable and exacerbation state definitions in the literature of bacterial presence in COPD. Each study used microbiological culture for detection.

Bacterial load has been less frequently explored, and this has principally involved the utilisation of quantitative culture techniques. Qualitative culture involves assessing whether a specific organism shows growth during culture, irrespective of load. Quantitative culture incorporates counting the number of colony forming units (CFU) growing on a plate and converting this into a value of CFU/ml (Miles et al. 1938). There are a number of advantages and disadvantages associated with quantitative culture. It is a relatively inexpensive method of identifying a diverse range of bacterial species, and also measures bacterial load. However, there is a delay of at least 24-48 hours before any colony growth is identified, potentially delaying any therapeutic response. Also, competition between bacterial species may result in some species not growing under culture conditions.

ATCC strains of the three typical airway bacteria were used as standards to provide reference quantification markers of bacterial load, for the quantitative PCR (Table 3.3). This methodology has been described in section 2.6.

Organism	ATCC Strain	DNA concentration (pg/ul)	CFU/ml
Haemophilus influenzae	10211	113500	1.26E+09
Moraxella catarrhalis	25238	147200	2.40E+10
Streptococcus pneumoniae	49619	18200	4.60E+09

Table 3.3. ATCC strains of the three typical airway bacteria were cultured in duplicate and DNA was extracted. This table shows DNA concentration (pg/µl) of neat samples for the three typical airway bacteria. This was then converted into CFU/ml measurements, using the culture data for reference.

From the standard curve established as described in section 2.7, the minimum load at which bacteria could be accurately quantified was able to be determined (Table 3.4).

Species	Lower detection limit (CFU/ml)
H. influenzae	1.25E+04
M. catarrhalis	2.07E+04
S. pneumoniae	6.40E+04

Table 3.4. Quantification limit for typical bacteria. Any samples which cross the cycle threshold below the lower detection limit are considered negative, as there is no positive control in the standard curve to verify their validity.

3.2 Subjects

A total of 202 patients from the London COPD cohort participated in the study, with data contributed between January 2007 and November 2012. Overall patient characteristics, in the stable state, are outlined in Table 3.5.

Characteristic	
Median age (range), years	70.5 (43-92)
Male gender (%)	64.5
Current smoker (%)	30.8
Median pack-year smoking history (IQR)	47 (30-70)
Mean FEV1 (SD), Litres	1.25 (0.5)
FEV1 % predicted (SD)	50.3 (18.9)
FEV1/FVC ratio (SD)	0.47 (0.1)
Mean FVC (SD), Litres	2.7 (0.9)

Table 3.5. Baseline physiological characteristics of the 202 patients.

3.2.1 Patient characteristics for culture & PCR comparison

Quantitative PCR was used in this study as the method for identifying presence and load of the typical bacteria. In a subset of patient samples, qPCR was compared against microbiological culture (culture previously performed by the Department of Medical Microbiology, Royal Free London Foundation Trust) to determine the

suitability of the methods (Table 3.6). Routine microbiological culture and qPCR for *H. influenzae*, *M. catarrhalis* and *S. pneumoniae* were compared in 439 sputum samples (214 stable, 119 exacerbation and 106 recovery samples) from 143 patients. Recovery samples were obtained in the 5 weeks following exacerbation.

Characteristic	
Median age (range), years	70.9 (47-92)
Male gender (%)	62.7
Current smoker (%)	28.2
Median pack-year smoking history (IQR)	47 (28-72)
Mean FEV1 (SD), Litres	1.2 (0.5)
FEV1 % predicted (SD)	48.4 (18.9)
FEV1/FVC ratio (SD)	0.45 (0.1)
Mean FVC (SD), Litres	2.6 (0.9)

Table 3.6. Stable-state clinical characteristics of 143 COPD patients examined in this analysis. No significant differences are seen between these and the patients described in Table 3.5.

Culture was performed by the Department of Medical Microbiology, Royal Free London Foundation Trust. Briefly, sputum was cultured onto suitable agar for 24 hours at 37^{o}C and 5% CO_2. Agar types used were Columbia blood agar (general growth media) (Ellner et al. 1966), Chocolate agar (growth of fastidious bacteria) (McLeod et al. 1927), MacConkey agar (growth of gram-negative bacteria) (Macconkey 1905), and COBA agar (growth of *Streptococcus spp.*) (Petts 1984). *S. pneumoniae* was identified through optochin sensitivity testing.

3.3 Results

3.3.1 Prevalence of typical airway bacteria using qPCR and routine microbiological culture

In 439 sputum samples, typical airway bacteria were identified on 241 occasions using qPCR, whereas the same three bacterial species were identified on only 106 occasions using culture ($p<0.001$). Of the 106 isolates detected by culture, 90 of these were also detected by PCR (20.5% of total; 85.1% concordance). Additionally, PCR detected typical airway bacteria in 151/439 (34.4% of total) sputum samples, which did not show typical airway bacterial presence by culture. A total of 16 isolates (3.6%) were detected by routine culture but were undetected by PCR.

A comparison of the two techniques was then performed examining each individual typical airway bacterial species, to confirm that the significant difference in the two methods was maintained across each of the three major typical airway bacteria. It was found that *H. influenzae* was detected in 28.5% of samples with PCR, compared with 15.0% of samples using culture ($p<0.001$); *S. pneumoniae* was found in 15.3% of samples with PCR versus 6.8% of samples with culture ($p<0.001$); and *M. catarrhalis* was detected in 11.2% of samples with PCR compared with 2.3% of samples with culture ($p<0.001$) (Figure 3.1).

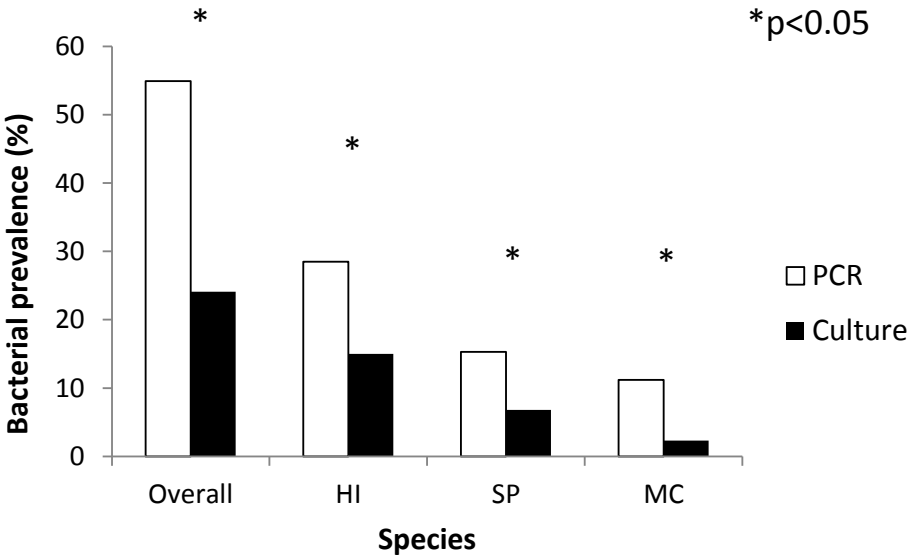


Figure 3.1. Comparison of culture and qPCR for the detection of three typical airway bacteria from 439 sputum samples. Species: HI = *Haemophilus influenzae*; SP = *Streptococcus pneumoniae*; MC = *Moraxella catarrhalis*.

3.3.2 Cases detected by PCR and culture

It was identified that there were a number of cases where both PCR and culture detected a pathogen isolate (n=90, 20.5%), where PCR detected a pathogen which culture did not detect (n=151, 34.4%) and where culture detected a pathogen which PCR did not detect (n=16, 3.6%).

3.3.3 Rate of detection of multiple bacterial species in samples by culture and qPCR

One of the limitations cited for culture is the potential for one micro-organism to outcompete other species during incubation (Hibbing et al. 2010). In order to combat this, various types of selective media are typically used to maximise the

possibility of detecting all clinically relevant pathogens. However, limited testing has previously been performed against molecular techniques.

In this study, qPCR was compared against culture in the same sample-set to see whether or not one method was more sensitive at detecting multiple typical bacterial species in a single sample (n=439). It was found that qPCR identified at least one typical airway bacterial species in 200/439 sputum samples (45.6%). Of these samples, 39 were found to have multiple species (19.5%; 8.9% of total sample set). On the contrary, culture detected typical airway bacteria in 95 of these 439 sputum samples (21.6%), of which eleven samples were shown to have multiple species (11.6%; 2.5% of total) (Figure 3.2).

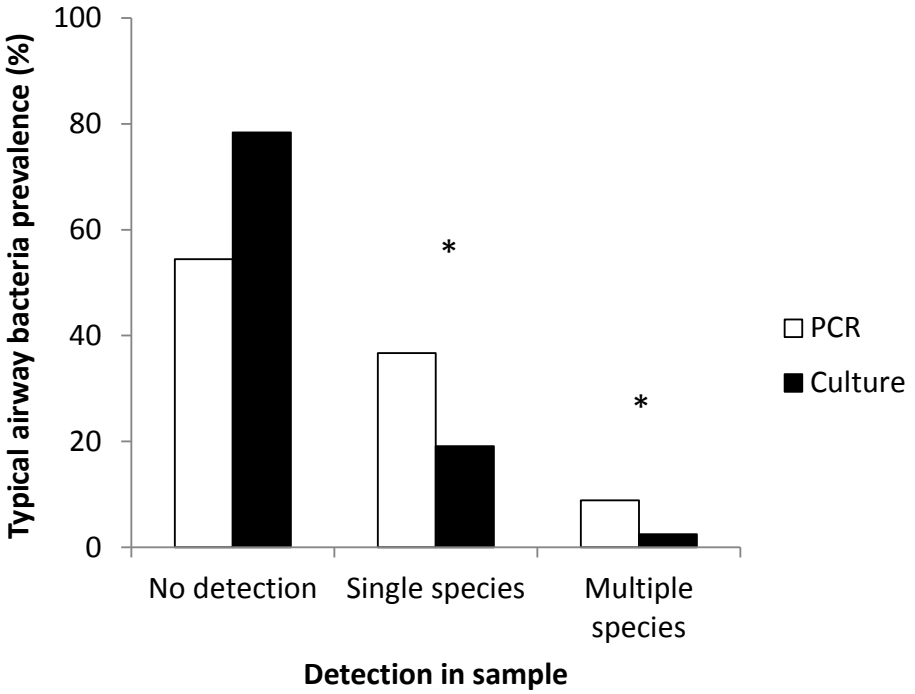


Figure 3.2. Detection rates of a single species or multiple species of typical airway bacteria (per sample), by PCR and by culture. PCR detected significantly higher levels of typical airway bacteria compared with culture. *p<0.05

Overall, examining the dataset of 439 samples, it was established that detection of multiple species was significantly increased by PCR compared with culture (8.9% vs. 2.5%; $p<0.001$). When the dataset is limited to those samples which had presence of typical airway bacteria, the difference in detection of multiple species by PCR and culture misses statistical significance (19.5% vs. 11.6%; $p=0.10$).

3.3.4 Prevalence of typical airway bacteria using a composite model compared with qPCR alone

Given that there were 16 cases (3.6%) where culture identified a bacterial isolate but PCR did not, it is prudent to examine the merits of a composite test (combination of culture and PCR) against PCR alone. PCR detected bacteria in 200/439 (45.6%) sputum samples, whilst the composite test detected bacteria in 207/439 (47.2%) sputum samples, representing a negligible difference (96.6% concordance; $p=0.64$). Of those isolates detected by culture alone, five were *H. influenzae* isolates, nine were *S. pneumoniae* isolates, and two were *M. catarrhalis* isolates.

3.3.5 Bacterial load in patients dependent on detection outcome

As considered in section 3.3.3, 200 samples were found to be positive for typical airway bacteria using PCR. From these 200 samples, bacterial species were detected by culture in 88 samples (44%). One possible reason for this discrepancy

may have been that growth on culture was dependent on bacterial load. In order to test this hypothesis, a comparison was performed examining bacterial load (quantified by PCR) between those samples positive on culture (and positive on PCR) with those samples negative on culture (positive on PCR).

It was found that the typical airway bacterial load was significantly different between the two groups: samples positive with both techniques had a mean (±SEM) cumulative bacterial load of $10^{7.9(\pm0.1)}$ CFU/ml, whilst samples positive on PCR alone had a mean cumulative bacterial load of $10^{7.1(\pm0.1)}$ CFU/ml ($p<0.001$).

Following this finding, bacterial load of individual species was then compared in those samples which were positive via culture and those samples which were negative with culture. All three species demonstrated higher PCR-determined bacterial load in culture-positive samples compared with those negative at culture. This difference was statistically significant for *H. influenzae* ($10^{7.3(\pm0.2)}$ vs. $10^{6.6(\pm0.2)}$ CFU/ml, $p=0.004$), where a five-fold higher species load was identified in culture-positive samples. A similar pattern was identified for *M. catarrhalis* ($10^{8.9(\pm0.2)}$ vs. $10^{7.7(\pm0.3)}$ CFU/ml; $p=0.001$), with a 16-fold higher species load found. However for *S. pneumoniae*, the difference was not significant ($10^{7.6(\pm0.2)}$ vs. $10^{7.2(\pm0.2)}$ CFU/ml; $p=0.159$) (Figure 3.3).

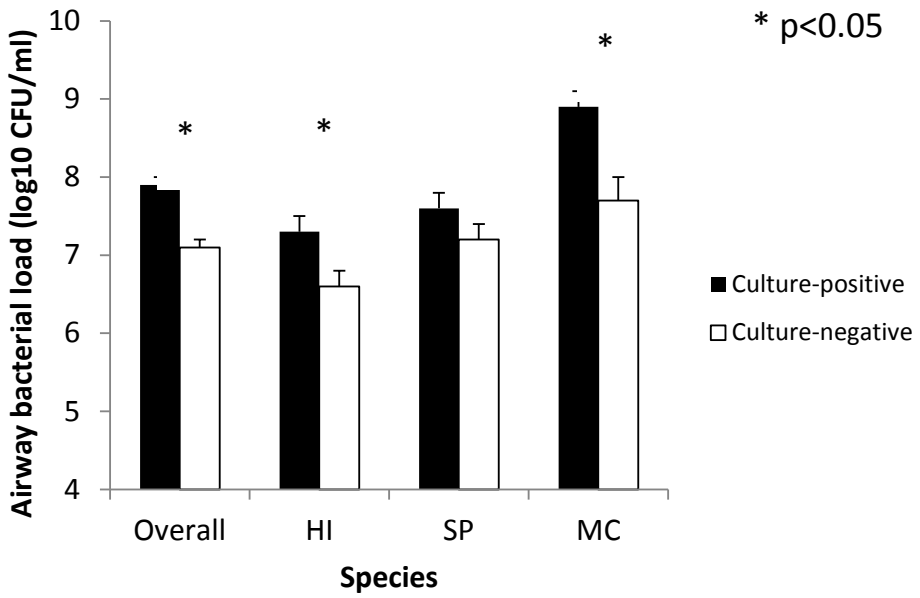


Figure 3.3. Bacterial load detected by qPCR segregated according to detection by culture. Overall, typical airway bacterial load is significantly higher in those samples which were detected by culture and PCR, compared with those samples in which detection was solely by PCR. When sub-divided by species type, this difference was maintained for HI and MC. Species: HI = *Haemophilus influenzae*; SP = *Streptococcus pneumoniae*; MC = *Moraxella catarrhalis*.

3.4 Discussion

In this study, quantitative PCR has been compared with microbiological culture in order to assess the most appropriate method to identify typical airway bacteria. Alternative methods for assessment such as microarray analysis were not considered for a number of reasons – microarray analysis for pathogen identification typically requires an initial PCR step for amplification of DNA (Stoughton 2005) and so would not provide higher detection rate compared to PCR alone; also, both PCR and microarray analysis equipment would be required resulting in considerably higher initial cost. One benefit of microarray analysis is that it would allow screening of more species than by PCR, but this was not a primary objective of the current study and so PCR was considered to be appropriate as the molecular method to compare against culture.

Molecular techniques such as PCR offer higher sensitivity than culture. The specificity of PCR is variable, dependent upon a number of factors involved in the setup of the PCR. For example primers and/or probes need to be shown to be specific to the target organism. There is also a possibility of PCR inhibitors being present in DNA extract, thereby limiting amplification when target DNA is present (Huggett et al. 2013). This study has attempted to overcome such limitations – the multiplex typical airway bacteria PCR has previously been validated by screening against a range of airway bacteria and viruses (performed by Dr Clare Ling and Julianne Lockwood, Department of Medical Microbiology). PCR inhibition was identified and corrected for by use of an IAC.

A previous study performed comparing PCR and culture in COPD (Curran et al. 2007) and a larger study in LRTI samples (Kais et al. 2006) identified that PCR was significantly more effective at detecting typical airway bacteria. The current investigation looked to validate a novel multiplex PCR assay as an appropriate method for bacterial identification.

The study performed by Curran and colleagues differed from the current methodology on a number of key parameters, meaning that it was important to validate the current technique. For example, the previous study had different gene targets to the ones used in this investigation. In particular, they targeted the *ply* (pneumolysin) gene for detection of *S. pneumoniae* – Kais and colleagues also targeted this gene. However, this gene has since been shown to not be a suitable target for routine use in *S. pneumoniae* detection due to non-specific binding (Abdeldaim et al. 2010). In the current study, non-specific amplification was overcome by targeting species-specific genes/DNA fragments – for *S. pneumoniae*, the spn9802 gene fragment was targeted, and this is known to be a more specific target, and suitable for routine use in the detection of *S. pneumoniae* (Abdeldaim et al. 2008; Abdeldaim et al. 2010). In the study by Kais and colleagues (Kais et al. 2006), they detected typical airway bacteria in 37.7% of LRTI samples using PCR, compared with 23.7% using culture, constituting an increase of 59.1%.

It has been demonstrated that quantitative PCR detects a higher number of typical airway bacteria than the current practice of routine microbiological culture. In this study typical airway bacteria were detected using PCR in 54.9% of samples, whilst

culture detected typical airway in 24.1% of samples. The samples examined for pathogens in this investigation were sputum samples. The use of sputum samples is due to the fact that they are non-invasive to the patient and easily collectible. A limitation of sputum is that it can be contaminated with upper airway bacteria during expectoration.

Other techniques which are often used in studies of lower airway bronchial colonisation (LABC) include PSB sampling and BAL fluid sampling. Both PSB and BAL fluid sampling involve invasive bronchoscopy in order to obtain the relevant sample. It can therefore be more difficult to obtain such samples, particularly during an acute exacerbation. Furthermore in a study examining the concordance between sputum and PSB sampling, it was found that there was high concordance between these two techniques (Soler et al. 2007). Taking all factors into account, sputum sampling is considered to be a suitable, non-invasive technique to assess LABC.

The primary advantages of qPCR demonstrated in this chapter are that it gives a higher rate of detection than culture and also that it gives an objective measure of bacterial load, rather than relying on operator colony counts characteristic of culture quantification, which may be liable to error.

Given the higher numbers of positive detections via PCR compared with culture, it was hypothesised that those samples which are detected by both methods have a higher bacterial load than those which were not detected by culture. Through this

investigation, it was found that there was a significantly higher bacterial load in those samples detected by both methodologies, at $10^{7.9}$ CFU/ml, compared with a load of $10^{7.1}$ CFU/ml in those samples which were not detected by culture. This constitutes an absolute load difference of six-fold and provides evidence which implicates lower bacterial load as a reason why culture may not detect all cases of typical airway bacteria presence.

Examining species individually, it was seen that for *H. influenzae* and *M. catarrhalis* there was a significantly higher bacterial load in those samples detected by both methods, compared to those detected by qPCR alone.

A small number of samples (3.6%) were found to be negative for typical airway bacteria using PCR but positive on culture. This may be due to a number of reasons, including operator error in the PCR, point mutation in the target gene preventing primer binding, PCR inhibition, mis-identification of organism on culture, or contamination of culture plates. A similar finding has been reported in a previous study examining PCR versus culture, with 4.8% of samples found to be negative with PCR and positive with culture (Kais et al. 2006). Chaidir and colleagues compared PCR with microbiological culture for the detection of *Mycobacterium* tuberculosis, finding that 8% of culture-positive results were negative using PCR (Chaidir et al. 2012). In the current investigation, using a composite model combining PCR and culture methods provided negligible additional benefit compared with using PCR alone.

It is important to consider that while qPCR is the most appropriate method to establish prevalence and load of typical airway bacteria as required by this study, microbiological culture still has a significant role to play in bacteriology. For example, culture allows antibiotic sensitivity testing to be performed in order to identify bacterial resistance profiles. Furthermore, those organisms which are rarely seen in COPD may not be routinely screened for using qPCR, and so again, may be identified through culture. Therefore, culture should by no means be wholly discounted from bacteriology assessment.

It is also worthwhile to consider that whilst culture solely detects viable bacteria, qPCR detects both viable and non-viable bacteria. This means that a subset of the qPCR-detected population may be non-viable bacteria (i.e. lysed bacterial cells with exogenous DNA). However, there are various inbuilt defences in the airways to remove such exogenous DNA (Palaniyar et al. 2005). Furthermore, it is currently unclear whether non-viable bacteria have any deleterious effects in their own right if they remain in the lungs, as endotoxins such as lipopolysaccharide may contribute to airway inflammation – this has previously been demonstrated in animal models (Okamoto et al. 2004).

A major aim of this project has been to assess the technical ability of qPCR for the detection of lower airway bacterial species in COPD sputum samples. To this end, it was compared in this study against the current standard technique of microbiological culture. It was found that PCR was significantly more effective at detecting bacteria compared to culture. This finding has previously been

demonstrated in a number of diseases (Dragsted et al. 2004; Boutaga et al. 2003; Kais et al. 2006; Curran et al. 2007). However, this is the first time it has been demonstrated in a study solely consisting of COPD patients.

Additionally, it was seen that in absolute numbers, qPCR detected multiple pathogens in samples more frequently than culture. This finding that qPCR is more effective than culture at detecting co-infection could have important clinical implications, as it would allow physicians to ensure that all pathogens have been eliminated, or at minimum, reduced below detectable levels.

3.5 Conclusion

Quantitative PCR is a more rapid method of detecting typical airway bacteria in sputum than routine microbiological culture, with greater detection rate also identified. This has been shown to be the case for each bacterial species being investigated. As such, subsequent analyses in this study will focus on results obtained by qPCR.

CHAPTER 4. **Prevalence and load of typical airway bacteria in COPD patients at both stable and exacerbated states of disease**

4.1 Introduction

In the stable state, the majority of patients are kept on maintenance therapy, involving use of a bronchodilator (LABA/LAMA) and/or inhaled corticosteroids. Occasionally, patients may suffer AE-COPD, during which symptoms either emerge or increase in severity, more than seen during day-to-day variations. Production of sputum varies in the COPD population. The sub-population of chronic bronchitis sufferers in COPD makes up 34% of the COPD population (Snoeck-Stroband et al. 2008), and chronic bronchitis is associated with chronic spontaneous sputum production for at least 3 months of the year, for two consecutive years. Conversely, patients who express a mainly emphysematous phenotype tend not to spontaneously produce sputum during stable disease. Many patients fall in between these two phenotypes and may occasionally expectorate sputum at the stable state (Kim & Criner 2013). During exacerbation, the vast majority of patients in the London COPD cohort spontaneously expectorate sputum. Exacerbations are a significant clinical experience in the life of a COPD patient, contributing to lung function decline and being associated with increased risk of myocardial infarction and mortality (Donaldson et al. 2010).

Presence of typical airway bacteria has been associated with exacerbations (Rosell et al. 2005; Monso et al. 1995). This is because prevalence of such bacteria is higher in patients at exacerbation than at the stable state. There is, however, presence of typical airway bacteria in a proportion of patients during stable state, with studies suggesting a rate of between 25% and 31% by microbiological culture

(Monso et al. 1995; Rosell et al. 2005; Zalacain et al. 1999). Patients who had typical airway bacteria detected (by culture) during stable state demonstrated higher levels of airway inflammatory markers compared to those without presence: the sputum levels of myeloperoxidase and interleukin-8 (IL-8) are known to be significantly higher in infected COPD patients than in non-infected patients (Bresser et al. 2000). The association of systemic inflammatory markers in stable and exacerbated COPD has previously been examined, and it has been shown that even at stable state, there are significantly higher levels of systemic inflammatory markers (TNF-α and IL-6) compared with healthy controls (Karadag et al. 2008). However, the relationship of systemic inflammation with presence or absence of typical airway bacteria in stable COPD has not previously been explored.

It is also of importance to establish whether the bacterial load changes in patients between the two states. Previously, higher bacterial load has been seen at exacerbation, when compared to the stable state (Rosell et al. 2005; Wilkinson et al. 2006b) and there is precedent from other disease where it has been shown that higher bacterial load is related to greater disease severity, including meningococcal disease (Hackett et al. 2002), pneumococcal pneumonia (Rello et al. 2009; Werno et al. 2012) and gastric inflammation (Varbanova & Malfertheiner 2011). However, in COPD, conflicting data suggests that bacterial load does not rise significantly at exacerbation (Sethi et al. 2007). In those studies, culture colony counting was used as the method to determine bacterial load. As demonstrated in Chapter 3, molecular techniques may provide a more appropriate method for the determination of bacterial load, and so in the current study, changes in bacterial

prevalence and load between the stable state and exacerbation were examined by qPCR.

4.2 Analysis of typical airway bacteriology in sputum from patients at either stable or exacerbation states

Previous studies have addressed the issue of bacterial load in different states of COPD. However, they were limited by one of a number of factors: the data involved unpaired analysis between stable and exacerbation states, meaning that there may have been a population bias and we cannot definitively say that the changes were caused by the change in state (Monso et al. 1995; Rosell et al. 2005); or intra-patient variations looked solely at those more severe exacerbations which result in hospitalisation (Papi et al. 2006). The current study therefore examined intra-patient variations in COPD patients, irrespective of the severity of the exacerbation. The prevalence and load of bacteria in COPD patients using molecular techniques has also been relatively little-studied, both in paired and unpaired analyses. As described earlier, quantitative PCR is more discriminatory than culture at detection of the commonly identified typical bacteria. It also allows for absolute quantification of bacterial load in the two states. Additionally, in a clinical setting, PCR offers a much more rapid turnaround time in identifying target organisms, compared to culture (Espy et al. 2006). The current study has utilised qPCR to assess changes in typical airway bacterial prevalence and load in COPD patients between stable and exacerbation states.

In the current study, patients expectorating sputum at either stable or exacerbation state had their typical airway bacterial levels assessed via qPCR. In cases where

patients were unable to spontaneously expectorate sputum, it was induced in consenting patients, as described in section 2.4.

4.2.1. Clinical assessment

Serum C-reactive protein (CRP) quantification was performed by the Department of Clinical Biochemistry (Royal Free London NHS Foundation Trust) to assess systemic inflammation, using Modular Analytics E 170 Module (Roche, burgess Hill, UK). Patients recorded inhaled corticosteroid dosage and usage daily on diary cards. Due to variations in dosage by different medications, a beclomethasone-equivalence dosage correction was applied, as previously described by our group (Hurst et al. 2006).

4.3. Results

4.3.1. Sample acquisition

In total, 544 stable state and 229 exacerbation state samples were analysed between January 2007 and October 2012, as part of the sample collection performed in the London COPD cohort. This involved a total of 202 patients, whose characteristics were previously described in Chapter 3 (table 3.5).

4.3.2. Characteristics of patients with paired state data

A sub-analysis of 61 patients, examining a pair of stable and exacerbation state sputum samples per patient, was performed to reduce bias which may occur when different patients are used to reflect changes in bacteriology in COPD states. This sub-analysis involved stable state samples being obtained less than 365 days prior to an exacerbation, with median (IQR) number of days separating stable state and exacerbation being 103 (60-148) days. Patient characteristics for these 61 patients are shown in Table 4.1.

Characteristic	
Median age (range), years	69.5 (47-83)
Male gender (%)	62.3
Current smoker (%)	26.2
Median pack-year smoking history (IQR)	43.5 (27.5-57)
Mean FEV1 (SD), Litres	1.18 (0.5)
FEV1 % predicted (SD)	46.8 (18.5)
FEV1/FVC ratio (SD)	0.44 (0.1)
FVC (SD), Litres	2.7 (1.0)

Table 4.1. Characteristics of 61 patients of the London COPD Cohort, who participated in a longitudinal study of typical airway bacterial presence in COPD. No significant differences are seen between these and the patients described in Table 3.5.

4.3.3. Typical airway bacterial prevalence

From a total of 544 stable state samples, 183 (33.6%) had presence of at least one typical airway bacterial species. Of 229 exacerbation samples, 122 (53.3%) showed such presence, a significantly higher prevalence rate than at the stable state ($p<0.001$) (Figure 4.1).

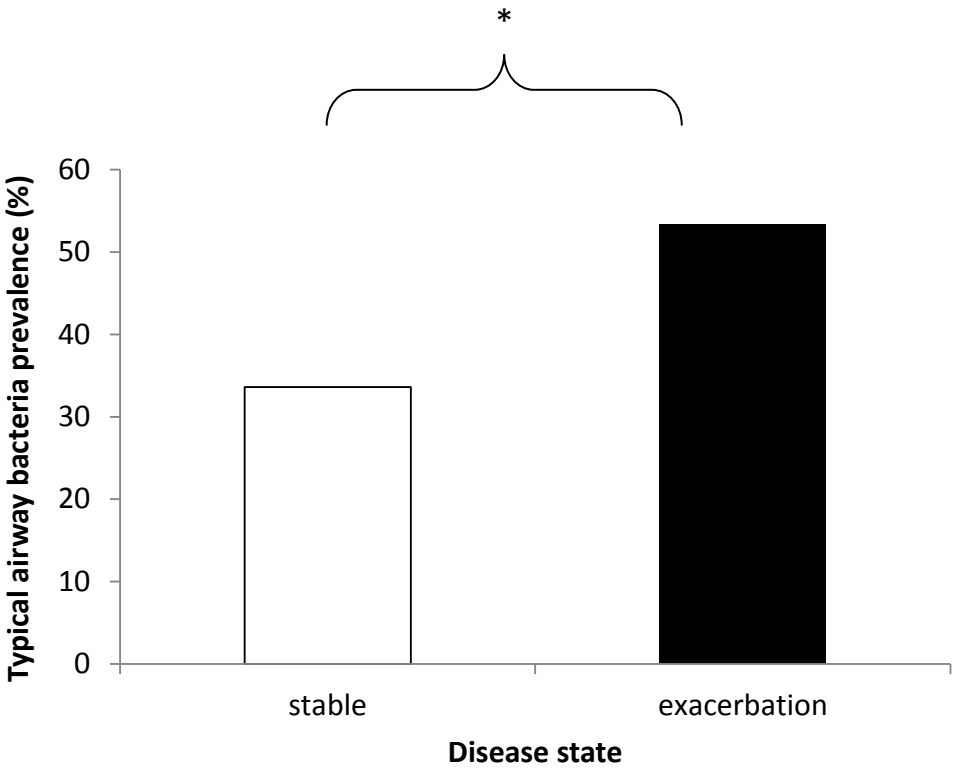


Figure 4.1. Comparison of typical airway bacteria prevalence at different phases of COPD. *p<0.05

On an individual species basis, there were statistically significant increases in prevalence rate of both *H. influenzae* ($p<0.001$) and *M. catarrhalis* ($p=0.003$). For *H.influenzae*, the prevalence increased from 20.0% at stable state, to 32.8% at exacerbation state. For *M. catarrhalis*, the prevalence increased from 8.5% to

15.7% (Figure 4.2). For *S. pneumoniae*, no significant increase was identified, with prevalence changing from 14.9% to 16.6% (p=0.55).

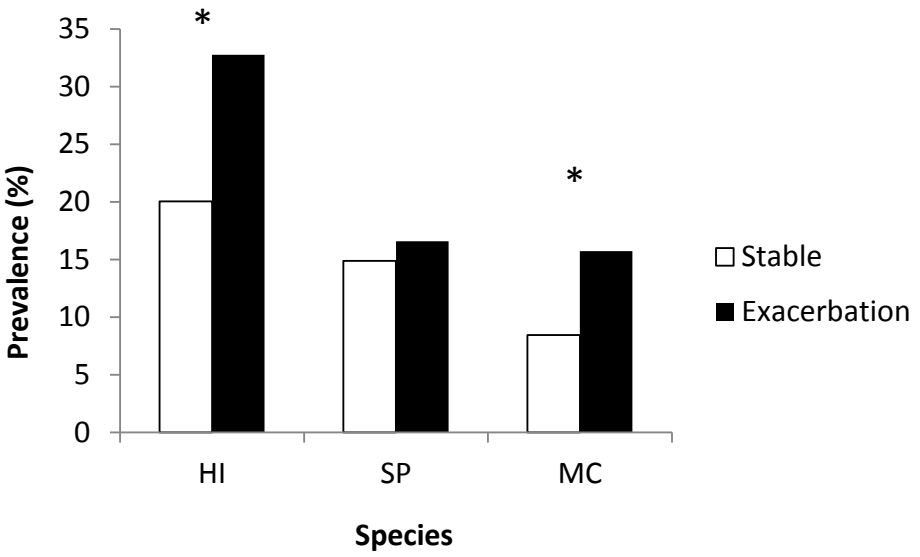


Figure 4.2. Prevalence of the three typical airway bacteria examined, at both stable and exacerbation states. HI = *Haemophilus influenzae*, SP = *Streptococcus pneumoniae*, MC = *Moraxella catarrhalis*. *p<0.05

4.3.4. Typical airway bacterial load

Specifically examining samples with typical airway bacterial presence (n=183 at stable state; n=122 at exacerbation), it was identified that bacterial load was significantly higher at exacerbation than at the stable state ($10^{7.8(\pm0.1)}$ vs $10^{7.2(\pm0.1)}$ CFU/ml; p=0.002), constituting a 4-fold higher typical airway bacterial load at exacerbation (Figure 4.3).

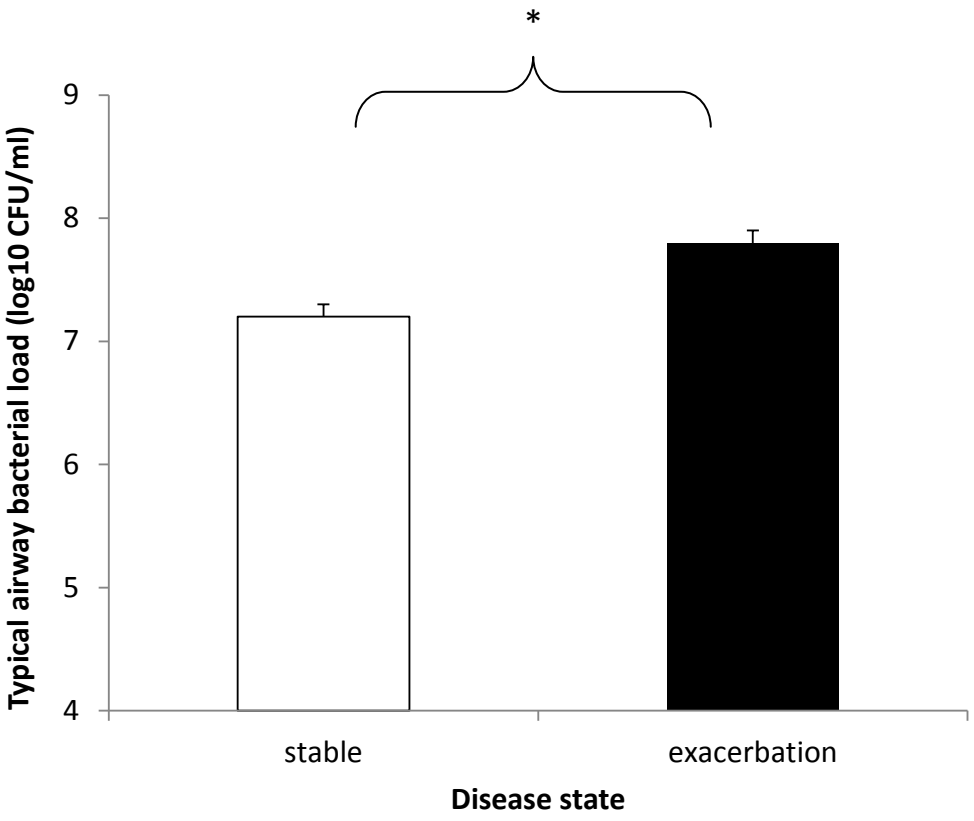


Figure 4.3. Mean (SEM) typical airway bacterial load in patients at stable and exacerbation states. *p<0.05

Analysis of the data with respect to the individual typical airway bacterial species showed that in patients testing positive for these species, there was significantly higher load of *H. influenzae* and *S. pneumoniae* at exacerbation compared to the stable state (Figure 4.4). For *H. influenzae*, mean load was $10^{6.7(\pm0.1)}$ CFU/ml in patients positive at stable state, whilst for patients positive for the organism at exacerbation the mean bacterial load was $10^{7.2(\pm0.2)}$ CFU/ml (p=0.018). In patients positive for *S. pneumoniae* during stable state, mean load was $10^{6.9(\pm0.2)}$ CFU/ml. At exacerbation, mean load for *S. pneumoniae*-positive patients was $10^{7.8(\pm0.2)}$ CFU/ml (p=0.002). No statistically significant difference was seen in the load of *M. catarrhalis* between the stable state and exacerbation. At stable state, the mean

M. catarrhalis load of patients positive for the bacterium was $10^{8.1(\pm0.3)}$ CFU/ml, whilst at exacerbation, mean bacterial load was $10^{8.3(\pm0.3)}$ CFU/ml (p=0.60).

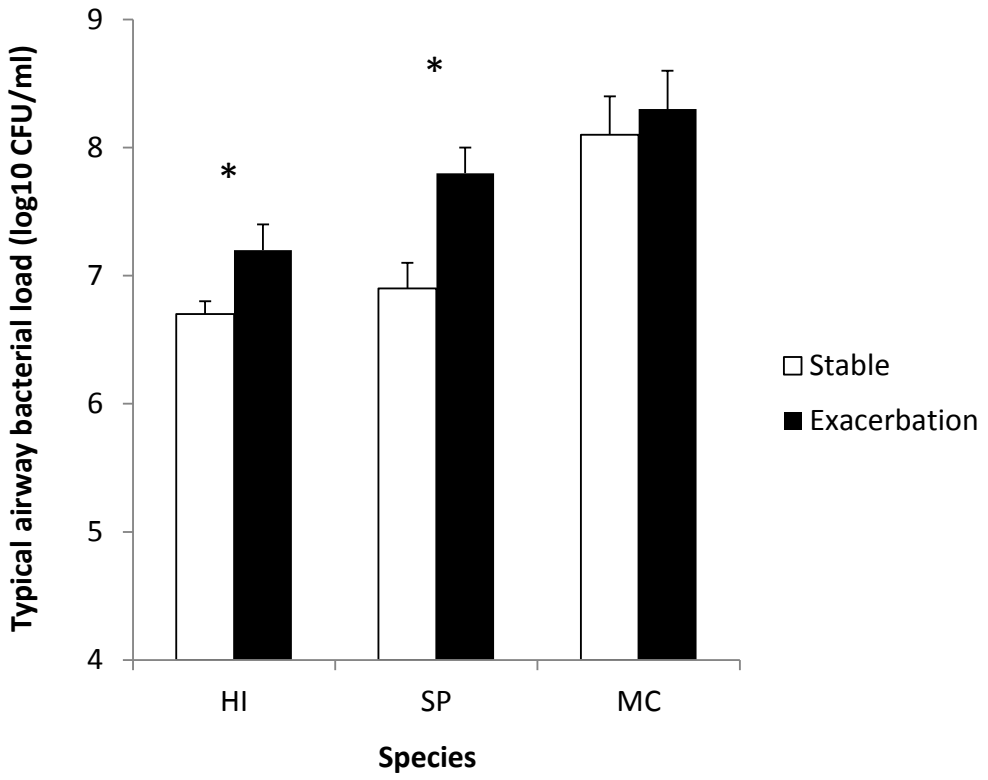


Figure 4.4. Mean airway bacterial load of the three bacterial organisms studied, at both stable and exacerbation states. HI = *Haemophilus influenzae*, SP = *Streptococcus pneumoniae*, MC = *Moraxella catarrhalis*. *p<0.05

4.4. Paired sub-analysis examining changes in bacterial prevalence and load between stable and exacerbation states

4.4.1. Typical airway bacteria prevalence in paired patients

Cumulative prevalence of typical airway bacteria was determined in 61 patients with paired stable and exacerbation state data. It was found that there was significantly higher cumulative prevalence of bacteria during exacerbation compared to stable state (68.9% vs 29.5%, $p<0.001$). In terms of number of patients carrying at least one typical airway bacterial species, 15/61 (24.6%) of stable-state samples showed presence of typical bacteria, whilst 34/61 (55.7%) of exacerbation-state samples showed typical airway bacterial presence ($p<0.001$) (Figure 4.5).

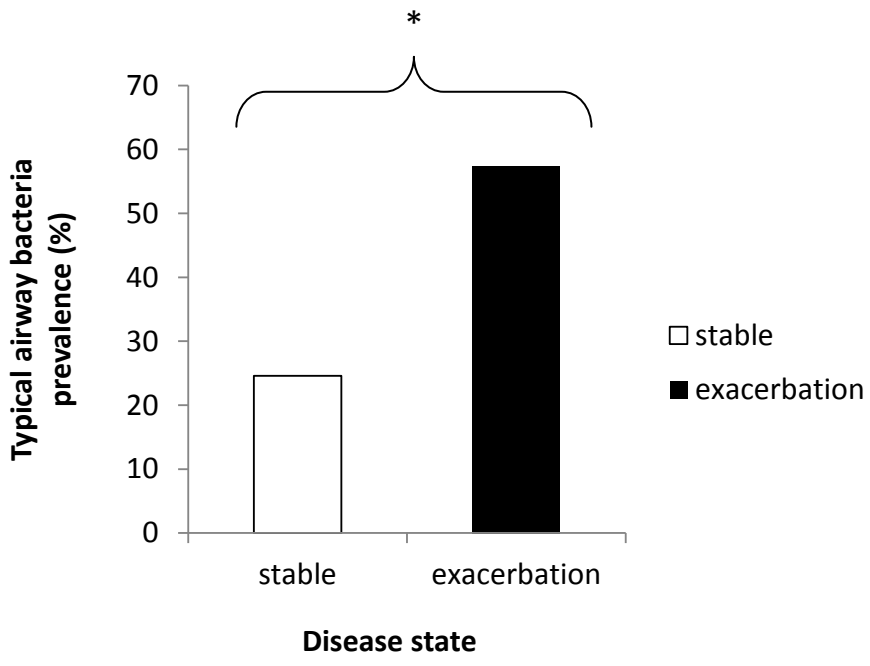


Figure 4.5. Prevalence of typical airway bacteria in 61 patients at both stable and exacerbation states. *p<0.05

Thirty-nine patients (63.9%) had typical airway bacterial presence at either stable state or exacerbation state. Of these, 10 patients (25.6%) showed presence at both sampling points, 5 patients (12.8%) showed presence only in the stable state, and 24 patients (61.5%) showed presence only in the exacerbation state.

Examining the changes in prevalence of individual typical airway bacteria between stable and exacerbation states in these 61 patients, it was found that there was a significant increase in prevalence in two of the three species under examination (Figure 4.6). During the stable state, 4 patients (6.6%) showed presence of *S. pneumoniae*. However, at exacerbation, 16 patients (26.2%) showed *S. pneumoniae* presence (p=0.003). This group of 16 patients included two of the four patients who had previously shown presence at the baseline state. Of the two patients who showed *S. pneumoniae* presence at baseline but not at exacerbation, one had acquired *H. influenzae* at exacerbation.

M. catarrhalis was present in the sputum of 2 patients (3.3%) during the stable state. At exacerbation, it was identified in 11 patients (18.0%) (p=0.008). Neither of the 2 patients with *M. catarrhalis* at the stable state were positive for the organism at exacerbation. One of those patients had *H. influenzae* at exacerbation, and the other patient had *S. pneumoniae* at exacerbation.

H. influenzae was seen in 12 patients (19.7%) at stable state, and in 15 patients (24.6%) during exacerbation, a non-significant change (p=0.51). Six of the 12 patients (50%) positive for *H. influenzae* at stable state were also positive with the

bacterium at exacerbation. Of the six patients who had *H. influenzae* solely at the stable state sample, two patients had *S. pneumoniae* at exacerbation. The remaining four patients had no typical airway bacteria detected at exacerbation.

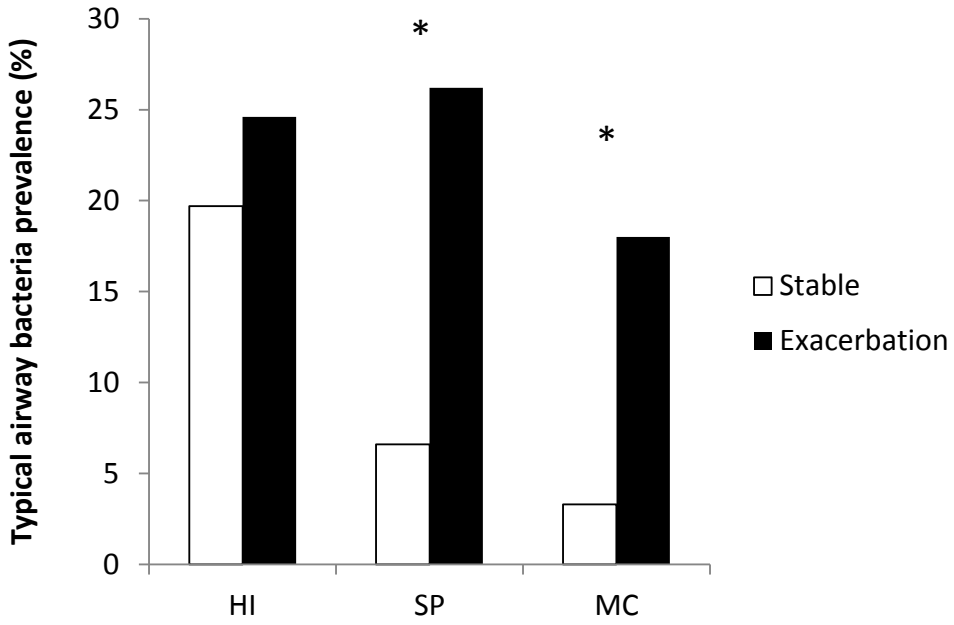


Figure 4.6. Prevalence of the three typical airway bacterial species in paired stable and exacerbation data. HI = *Haemophilus influenzae*, SP = *Streptococcus pneumoniae*, MC = *Moraxella catarrhalis*. *p<0.05

4.4.2. Typical airway bacterial load in paired patients

We were then able to compare changes in the bacterial load in these patients from whom we had paired stable and exacerbation state samples. The fifteen patients who showed typical airway bacterial presence in the stable state had a mean bacterial load at that point of $10^{7.4(\pm0.5)}$ CFU/ml. The 34 patients with presence in the exacerbation state had a mean bacterial load of $10^{8.3(\pm0.2)}$ CFU/ml.

Ten patients (16.4%) were positive for typical airway bacteria at both stable and exacerbation states. Typical airway bacterial load was significantly higher in these patients at exacerbation state compared with stable state, $10^{8.6(\pm0.2)}$ CFU/ml versus $10^{7.2(\pm0.6)}$ CFU/ml (p=0.02) (Figure 4.7).

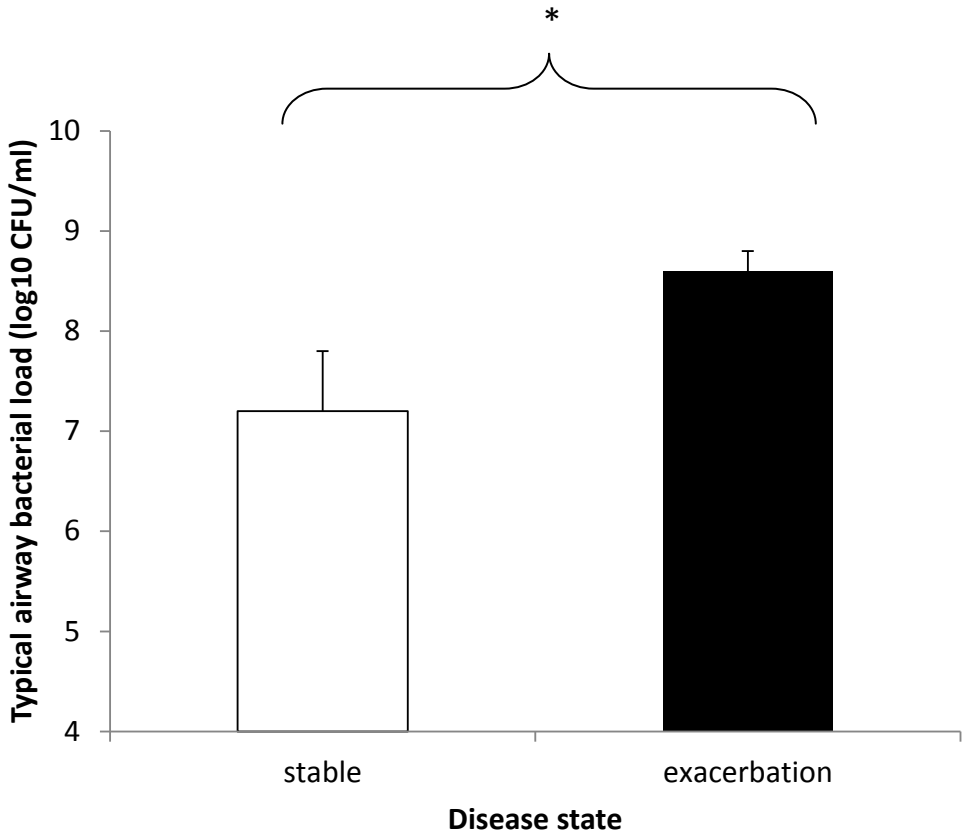


Figure 4.7. Mean typical airway bacterial load in ten patients who had typical airway bacteria both at stable and exacerbation states. *p<0.05

4.4.3. Effect of antibiotic therapy

In the paired analysis, a comparison was performed to determine whether antibiotic therapy in the intervening period may be having an effect on bacterial prevalence and load at the subsequent exacerbation. Antibiotic therapy data was

available on 40/61 patients. At exacerbation it was found that 15 patients had taken antibiotic therapy in the intervening period, of whom 9 (60%) showed presence of typical airway bacteria at exacerbation. Twenty-five patients had no evidence of antibiotic therapy usage in the intervening period. Of these 25 patients, 13 (52%) showed typical airway bacterial presence at exacerbation. This difference in prevalence was not statistically significant (p=0.62).

Furthermore, there was no significant difference in bacterial load at exacerbation, regardless of antibiotic therapy (p=0.54).

4.4.4. Changes in lung function between states

Spirometry data measuring lung function was available in 48/61 patients at both stable and exacerbation states. It was found that FEV_1 decreased by a median of 9.4% at exacerbation compared to the prior stable state.

It was also seen that presence of *H. influenzae* at exacerbation was associated with a poorer outcome in lung function compared to exacerbations which did not have *H. influenzae* presence (-16.1% vs 1.7%; p=0.015).

4.5.Systemic inflammation at stable state and at exacerbation

CRP data was available for 164 (30.1%) stable state samples and 69 (30.1%) exacerbation samples. It was found that median (IQR) systemic inflammation, as measured by serum CRP, was significantly higher at exacerbation compared to the stable state: 10.0 (3-46) versus 3 (1-7) mg/L (Figure 4.8) (p<0.001).

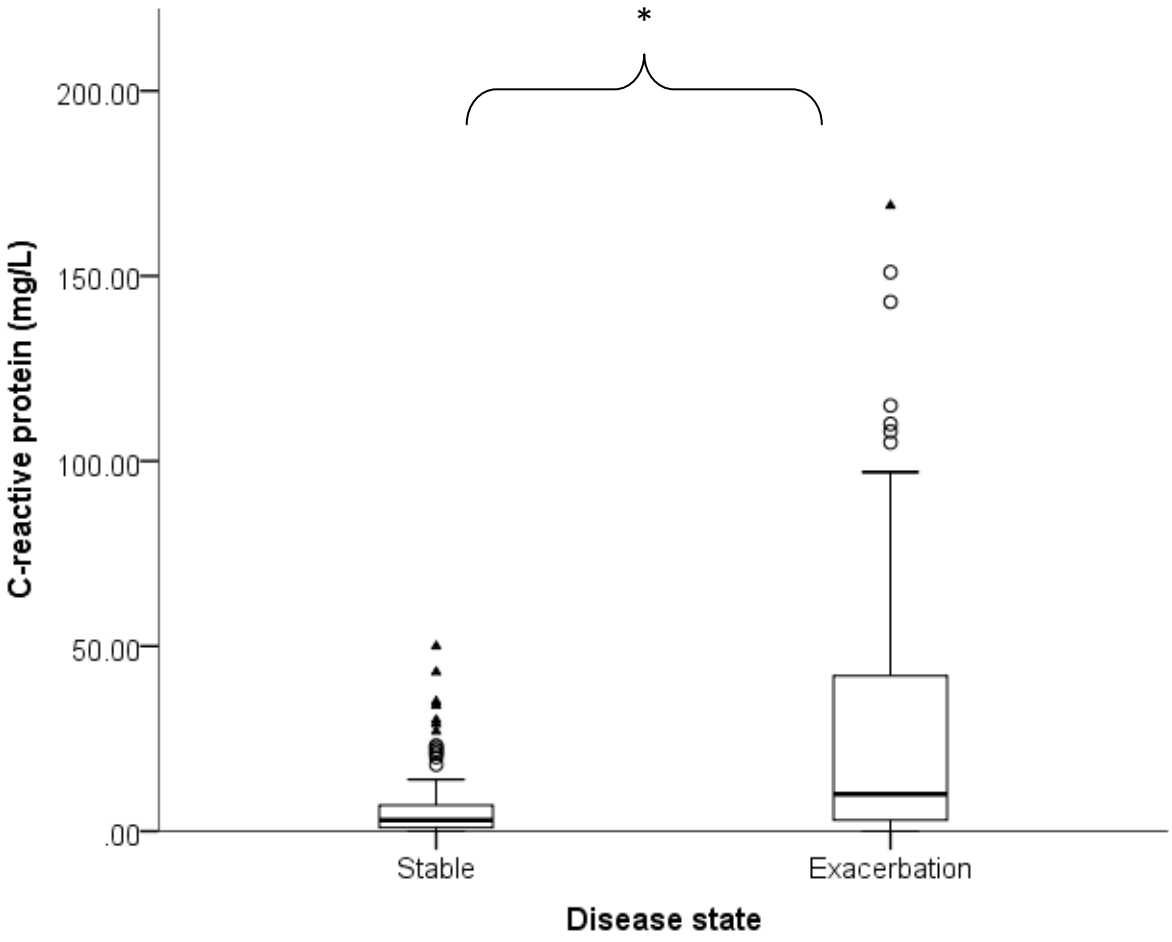


Figure 4.8. Median (IQR) levels of C-reactive protein at stable and exacerbation states of COPD. The median level of CRP is significantly higher at exacerbation than at stable state. Outliers shown with a circle are >1.5 times outside the IQR; Outliers shown with a triangle are >3 times outside the IQR. *p<0.05

In order to determine whether systemic inflammation was related to bacterial prevalence and load at exacerbation, CRP levels were analysed in available samples (Table 4.2).

Typical airway bacterial presence	CRP data available (n)
No presence	23
Presence of *H. influenzae* alone	17
Presence of *S. pneumoniae* alone	10
Presence of *M. catarrhalis* alone	7

Table 4.2. Sputum samples obtained at exacerbation, with both CRP and PCR data available.

Comparing those exacerbation samples which had presence of one typical airway bacterial species (n=34) with those which had no presence (n=23), there were significantly higher median (IQR) levels of CRP associated with those samples which had bacterial presence (17.5 (5.5-51.3) versus 3 (2-17) mg/L; p=0.009). It was also found that median CRP level was found to be highest in those patients who had presence of *H. influenzae,* at 26.0 (7.5-69) mg/L. This was significantly higher than in samples with no typical airway bacterial presence (p=0.004).

S. pneumoniae had a median CRP level of 12.5 (1.75-68.5) mg/L whilst *M. catarrhalis* had a median level of 10 (6-42) mg/L (Figure 4.9). Neither of these CRP

values differed significantly either from each other (p=0.96) or from that seen with *H. influenzae* presence (p=0.51 & p=0.46, respectively).

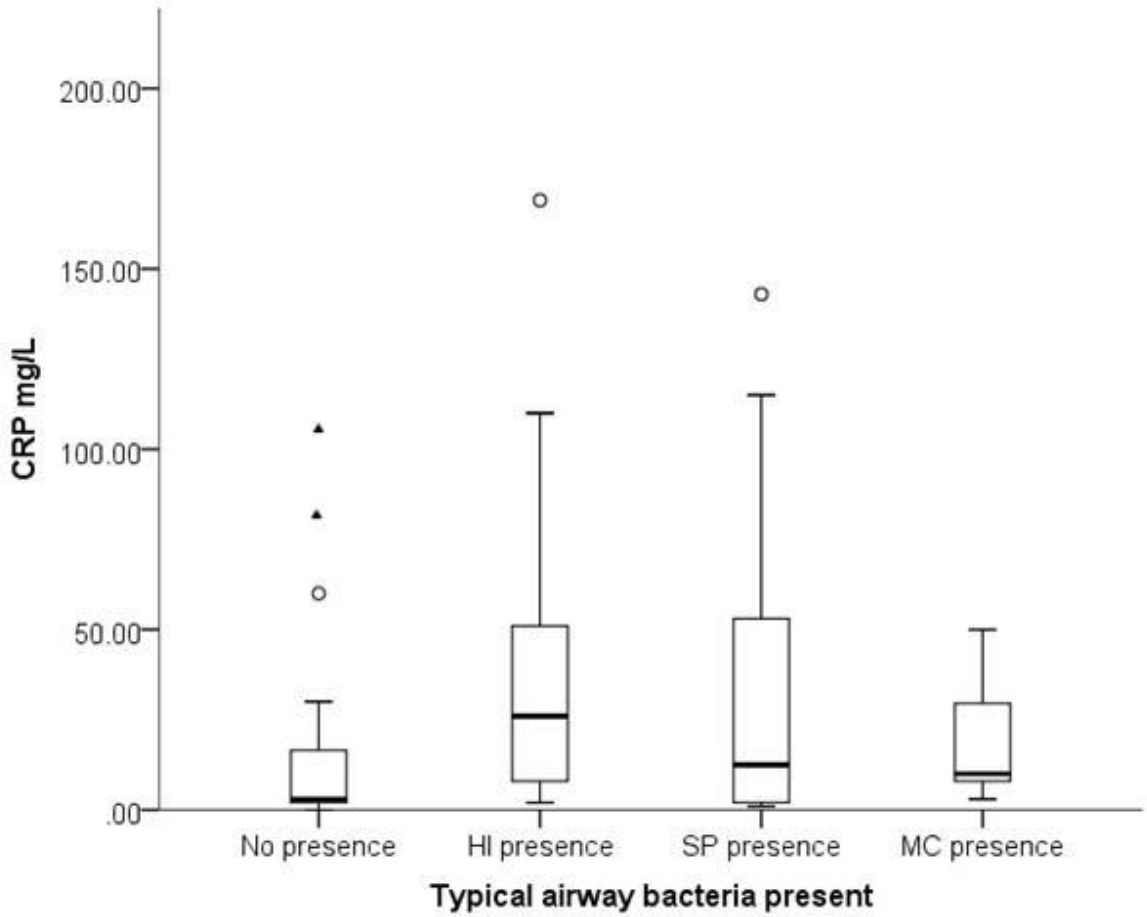


Figure 4.9. Median CRP levels of exacerbating COPD patients, separated into presence or absence of typical airway bacterial species. Outliers shown with a circle are >1.5 times outside the IQR; Outliers shown with a triangle are >3 times outside the IQR. *p<0.05. HI = *Haemophilus influenzae*, SP = *Streptococcus pneumoniae*, MC = *Moraxella catarrhalis*

Twelve of the 61 patients (19.7%) with paired stable and exacerbation state samples also had levels of CRP recorded at both stages. It was identified that in 11/12 patients (91.7%), CRP was higher at exacerbation compared to stable state. As the CRP data was normally distributed, mean (SEM) values of CRP at stable state and exacerbation were compared. Levels of CRP were significantly higher at

exacerbation compared to the stable state: 44.4 (±15.6) versus 8.7 (±2.5) mg/L, p=0.037 (Figure 4.10).

Additionally, it was established that in 8 of the 11 patients (72.8%) in whom CRP was higher at exacerbation, bacterial load was also higher at exacerbation – of the other three patients, two showed no signs of typical airway bacterial presence at either stable or exacerbation state, and one showed a fall in load from $10^{8.1}$ CFU/ml at stable state to $10^{7.5}$ CFU/ml at exacerbation state.

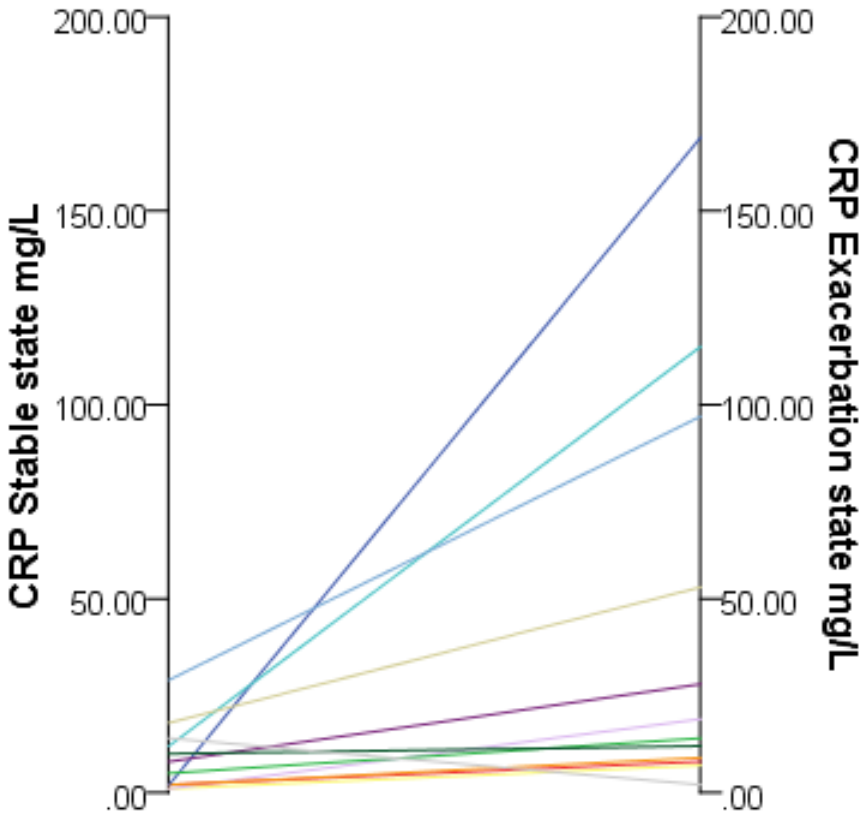


Figure 4.10. Changes in CRP values in 12 patients with paired stable state and exacerbation state data

4.5.1. Exacerbation symptoms and typical airway bacteria

Sputum purulence was measured in 206/229 exacerbations (90.0%). Typical airway bacteria were detected in 109 of these samples (52.9%) by qPCR. Typical airway bacterial load was found to be significantly higher in those samples which were associated with an increase in sputum purulence compared with those which did not show an increase ($10^{8.0(\pm0.2)}$ vs $10^{7.1(\pm0.3)}$ CFU/ml, p=0.01) (Figure 4.11). No significant difference was identified in typical airway bacterial load between samples with increase in sputum volume (n=76) and those without an increase in sputum volume (n=33) ($10^{7.6(\pm0.2)}$ vs $10^{8.1(\pm0.3)}$ CFU/ml; p=0.14).

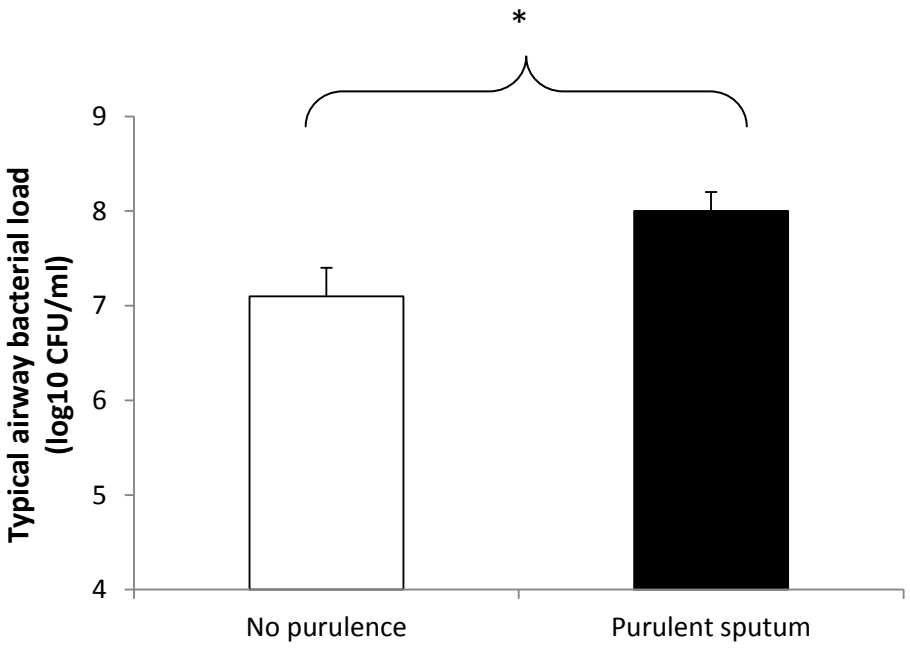


Figure 4.11. Typical airway bacteria load split by presence or absence of purulent sputum at exacerbation. Significantly higher load of typical airway bacteria was seen in purulent sputum. *p<0.05

One hundred and nine samples (52.9%) exhibited presence of typical airway bacteria, whilst 97 (47.1%) did not. Of those samples with typical airway bacterial prevalence, 79 (72.5%) showed an increase in sputum purulence, whilst 60 (61.9%) samples, which did not have typical airway bacteria detected, showed an increase in sputum purulence. This difference was not statistically significant (p=0.10)

4.6. Discussion

This study investigated prevalence of typical airway bacteria, and the load of these species, in the airways of COPD patients, measured at both stable state and during an acute exacerbation, when patients suffer deterioration in symptoms. Previous studies exploring this field have investigated a number of different facets of typical airway bacterial presence in COPD patients. Presence of airway bacteria has previously been described as colonisation, when identified in stable COPD, and as infection when identified in acute exacerbations (Parameswaran et al. 2011; Pomares et al. 2011). However, the current study has eschewed such definitive descriptions, and any detection of typical airway bacteria is described as bacterial presence, with the rationale for this being that the precise role of bacteria in the stable and exacerbation states of COPD is not clearly defined.

Bacteria are considered to be a major contributor to exacerbations in COPD (Beasley et al. 2012; Sethi 2010). Rosell and colleagues compared bacterial prevalence and load in patients with stable and exacerbated COPD, examining a Spanish population (Rosell et al. 2005). They identified significant increases in both bacterial prevalence and load at exacerbation.

Looking specifically at the three typical airway bacteria most frequently identified in COPD patients, the Spanish study found a prevalence rate of 29.8% was seen at stable state, compared with 44.2% at exacerbation (Rosell et al. 2005). However, this was determined solely by performing culture analysis, and looking at different

patients in each disease state. Bacterial load was described discretely rather than in a continuous manner i.e. samples were typed as having greater than 10^2, 10^3 or 10^4 CFU/ml. The implication for this on the data are that similar bacterial loads may be classed in different segments if some fall just below the threshold and others are slightly above the threshold. The current study utilised the actual bacterial load to avoid any bias that may arise by choosing arbitrary threshold units.

In the current chapter, it has been confirmed that typical airway bacteria presence does increase significantly between stable state and exacerbation. Further analysis is important to confirm whether this increase is driving such exacerbation.

An important component of the research performed in this chapter involved using both paired and unpaired data. Unpaired analysis comparing differences in the bacterial population between stable and exacerbation states of COPD has been utilised in the key literature in this field (Rosell et al. 2005; Monso et al. 1995). This is in large part due to the difficulties involved in conducting longitudinal studies, although these challenges were overcome in the current investigation. This assessment found that patients were more than twice as likely to have typical airway bacterial presence at exacerbation as at stable state. Furthermore, in those patients who had presence of typical airway bacteria at both stable state and at exacerbation, there was a mean increase in bacterial load of 25-fold at exacerbation. This confirms that typical airway bacteria are associated with COPD exacerbations, and indicates that bacterial load may be a critical factor in contributing to exacerbations of COPD.

The current study also shows that presence of *H. influenzae* at exacerbation is associated with a marked decrease in FEV_1 from stable state, which is not seen in those exacerbations without *H. influenzae* presence. This is the first time that such a finding has been conclusively demonstrated, and confirms a trend previously suggested in the London COPD cohort (Wilkinson et al. 2006b). The potential importance of this discovery is that it may allow antibiotic therapy to particularly target those exacerbations confirmed to be associated with *H. influenzae*. No such patterns were identified with the other typical airway bacterial species. A recent phase 2 clinical trial has investigated the use of oral inactivated NTHi as a mechanism of vaccinating against strains of NTHi which may contribute to COPD exacerbations (Tandon et al. 2010). The trial demonstrated that use of this oral vaccine reduced the rate of moderate-to-severe COPD exacerbation by 63%, and also reduced the mean duration of episodes from 22.7 days to 14.3 days. It is therefore feasible that *H. influenzae* could be specifically targeted to reduce exacerbation frequency and length.

There was no evidence to suggest that antibiotic therapy in the period between stable state and exacerbation impacted on bacterial prevalence or load at exacerbation. However, the sample set was relatively small and so it may have been underpowered to detect any differences. Daily diary cards provided records of patient symptoms at the time of exacerbation - previous work has indicated that sputum purulence and colour may be a surrogate marker for bacterial infection (Miravitlles et al. 2012; Stockley et al. 2000). In the current study, it has been demonstrated that load of typical airway bacteria is higher in patients with purulent

sputum than those without purulent sputum. However, it is also important to note that 27.5% of samples with presence of typical airway bacteria did not show an increase in sputum purulence, implying that sputum purulence alone is not the most appropriate method for identification of typical airway bacterial presence in COPD.

It has been shown that both bacterial prevalence and load increase significantly at exacerbation compared to stable state. It was therefore necessary to establish whether these increases are associated with changes in the inflammatory response. Using C-reactive protein (CRP) as a surrogate marker for systemic inflammation, this response was assessed. Systemic inflammation was shown to increase significantly at exacerbation compared to stable state. Of 12 patients for whom paired stable and exacerbation state CRP data was available, 11 (92%) showed higher CRP levels than at stable state. This is to be expected given that exacerbation represents a worsening of the disease, and this would typically manifest itself through an increased inflammatory response. It was also demonstrated in this study that exacerbations associated with typical airway bacteria have significantly higher CRP levels compared to other exacerbations, and this has also recently been confirmed by a Chinese investigation (Peng et al. 2013).

Presence of *H. influenzae* was found to be correlated with the highest rate of systemic inflammation, although both *S. pneumoniae* and *M. catarrhalis* also exhibited higher median CRP values compared with exacerbations which had none

of these typical airway bacteria detected. The findings of the previously-described clinical trial with the oral inactivated *H. influenzae* vaccine showed a significant reduction in moderate-to-severe exacerbations and shorter duration of episodes (Tandon et al. 2010), and, coupled with the current study, this would indicate that *H. influenzae* may be driving the inflammatory process, rather than the inflammatory process paving the way for *H. influenzae* infection. There is also precedent in cystic fibrosis where it was previously shown that *H. influenzae* was an important cause of inflammation, being associated with macrophage and neutrophil infiltration into the lungs (Armstrong et al. 1995). It has been illustrated in a number of studies that airway inflammation is also associated with airway bacterial presence in COPD. In 2000, Hill and colleagues found that in stable chronic bronchitis patients, airway inflammation was strongly correlated with airway bacterial load (Hill et al. 2000), and it has also been shown that the presence of so-called 'potentially pathogenic micro-organisms' is associated with higher levels of neutrophils, with such neutrophils contributing to the inflammatory response (Soler et al. 1999).

In conclusion, the evidence in this chapter has demonstrated that typical airway bacteria prevalence and load increases significantly from stable COPD to exacerbated COPD, and this is associated with a significant increase in CRP, a biomarker of systemic inflammation. At exacerbation, presence of typical airway bacteria was found to be associated with significantly higher levels of CRP than those exacerbations in which these bacteria were not detected. Presence of *H. influenzae* at exacerbation was associated with a particularly high CRP level,

suggesting that therapy targeting *H. influenzae* may be particularly beneficial to reduce exacerbation severity.

CHAPTER 5. **The relationship between typical airway bacterial load and clinical outcomes in stable COPD patients**

5.1 Introduction

As reported in Chapter 4 of the current study, 24.6% of stable-state samples exhibited presence of these typical airway bacteria. It has previously been considered that bacteria have little effect in contributing to exacerbations (Hirschmann 2000). Hirschmann discussed a number of concerns involved in the role of bacteria in COPD. He highlighted that previous research used unpaired analysis to demonstrate an increase in bacterial presence at exacerbation compared with stable state, and this made interpretation uncertain. This uncertainty has been answered in Chapter 4, where it was demonstrated that there is an increase in the prevalence rate of typical airway bacteria when examining the same patients at stable and exacerbation states. Hill and colleagues have also shown that during stable COPD, airway inflammation is higher in those patients who have airway bacterial presence (Hill et al. 2000). Specifically, the authors found that airway bacterial load was correlated with levels of sputum myeloperoxidase (a surrogate marker for neutrophilia), neutrophil chemoattractants and elastase.

The range of data collected in the London COPD cohort (Table 5.1) allows a number of clinical outcomes to be compared during the stable state.

Parameters
FEV1
FVC
Height
Age
Smoking status
Therapy regimens
qPCR bacterial loads
Microbiological culture
CRP

Table 5.1. Parameters available in the London COPD Cohort database.

In stable COPD, patients are often on maintenance therapy to treat the chronic symptoms of the disease. Inhaled corticosteroid (ICS) therapy is frequently prescribed to patients as a maintenance therapy in COPD, and it has been shown to control both airway and systemic inflammation in stable COPD patients (Confalonieri et al. 1998; Sin et al. 2004). The TORCH (Trial study ID: SCO30003) and INSPIRE (Trial study ID: NCT 00361959) randomised controlled trials have identified that ICS usage is related to higher rate of pneumonia development in COPD patients (Calverley et al. 2007; Wedzicha et al. 2008; Calverley et al. 2011). However, no mechanistic rationale for such a finding has so far been postulated. In the current

study, it was hypothesised that bacterial load is associated with ICS dosage in stable COPD patients.

It is known that at exacerbation, systemic inflammation is significantly greater than at stable state, as measured by CRP, a biomarker of systemic inflammation (Perera et al. 2007) – this was confirmed in Chapter 4 of the current study. In this chapter, it has been investigated whether systemic inflammation was also greater in patients with typical airway bacterial presence, than in those without, during the stable state. The importance of this is that it will help inform whether or not typical airway bacteria contribute to systemic inflammation at stable state.

5.2 Patient characteristics

A total of 90 patients of the London COPD cohort provided 164 stable state samples that also had levels of CRP recorded at the time of sampling (Table 5.2) – these patients represent a sub-population of the patients described in table 3.5.

Characteristic	
Median age (range), years	70.4 (47.3-92.2)
Male gender (%)	64.4
Current smoker (%)	32.2
Median pack-year smoking history (IQR)	47.0 (28.8-63.2)
Mean FEV1 (SD), Litres	1.3 (0.5)
FEV1 % predicted (SD)	52.6 (17.6)
FEV1/FVC ratio (SD)	0.48 (0.1)
FVC (SD), Litres	2.8 (0.8)

Table 5.2. Characteristics of 90 patients of the London COPD Cohort, who participated in a longitudinal study of typical airway bacterial presence in stable COPD and in whom CRP data was also available from the stable state sample. No significant differences are seen between these and the patients described in Table 3.5.

For ICS data analysis, beclomethasone-equivalent dosage conversion was performed to directly compare different types of ICS. Budesonide conversion involved multiplying the original dose by 0.8 to get a beclomethasone-equivalent dose, whilst fluticasone dose was multiplied by 2.0 for the same equivalence, as previously performed by our group (Hurst et al. 2006).

5.3 Results

5.3.1 Systemic inflammation in stable COPD

Of the 544 stable state samples examined in Chapter 4, 164 samples also had CRP data recorded. Overall, median (IQR) CRP data for these stable samples was found to be 3 (1-7) mg/L, as reported in Chapter 4. No clinically significant difference was identified in CRP data based on detection of typical airway bacteria: The median CRP value for samples negative for bacteria (n=106) was 3 (1-7) mg/L, and for samples positive for bacteria (n=58) it was 2 (1-7.25) mg/L. The CRP levels for patients with presence were subdivided according to species (Table 5.3).

	Median CRP (IQR) mg/L
SP positive only (n=17)	2 (1-4)
HI positive only (n=20)	3.5 (1-8.5)
MC positive only (n=8)	2 (1-8)
Multiple (n=13)	4 (2-17.5)

Table 5.3. Median CRP of stable state patients exhibiting presence of at least one typical bacterial species. HI = *Haemophilus influenzae*, SP = *Streptococcus pneumoniae*, MC = *Moraxella catarrhalis*. Multiple species relates to samples which contain more than one type of typical airway bacterial species.

5.3.2 Bacterial load and systemic inflammation

Having identified that bacterial presence alone is not associated with systemic inflammation in the stable state, analysis was performed to assess whether or not the load of bacteria influenced systemic inflammation. It was found that cumulative typical airway bacterial load (which represents the total typical airway bacterial load in a sample, following summation of each individual species load) positively correlated with systemic inflammation (rho=0.30; p=0.02) (Figure 5.1).

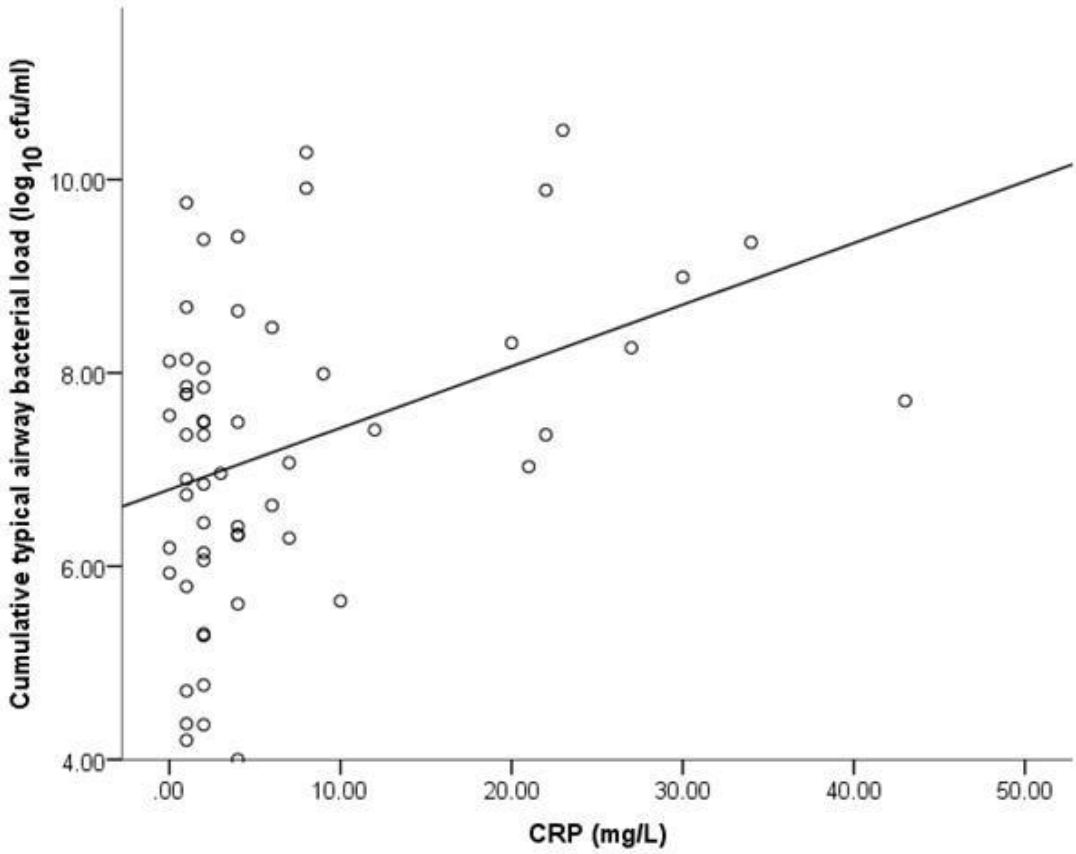


Figure 5.1. Relationship of typical airway bacterial load and C-reactive protein (CRP) (rho=0.30; p=0.02).

5.3.3 Systemic inflammation and airflow limitation

Given that there was a wide variation in CRP values for stable COPD patients, the sample population was assessed to determine whether there was a relationship between systemic inflammation and airflow limitation, measured by percentage predicted of expected FEV_1 (FEV_1% predicted). Airflow limitation and CRP data was available on 158 (96.3%) samples.

It was found that there was no correlation between systemic inflammation and airflow limitation (rho=-0.02; p=0.80). Furthermore, there was no difference in systemic inflammation across different GOLD stages (p=0.870) (Figure 5.2).

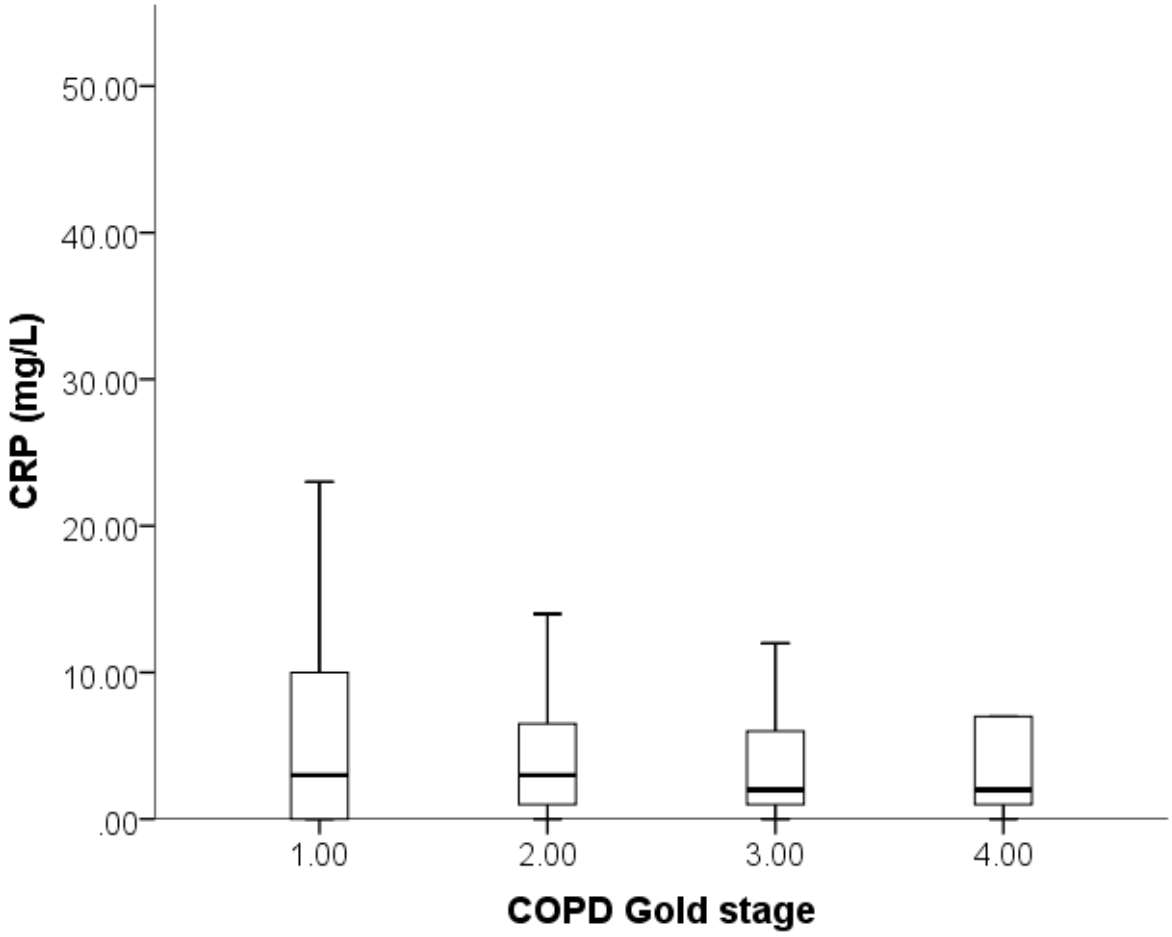


Figure 5.2. Correlation of CRP levels with GOLD stage.

5.3.4 Airflow limitation and typical airway bacteria at stable state

Typical airway bacterial load was previously found to be associated with systemic inflammation, in section 5.3.2. This load was further assessed to determine whether it impacted upon lung function on stable COPD patients (n=158). Initial analysis was performed to compare airflow limitation between those patients with typical airway bacterial presence (n=54, 34.2%) and those without (n=104, 65.8%).

No significant difference was identified in FEV_1% predicted in those patients with typical airway bacterial presence (FEV_1 50.4% predicted) and those patients without presence (FEV_1 55.4% predicted) (p=0.12). The 54 patients with typical airway bacterial presence were further assessed to see whether the bacterial load was associated with severity of airflow limitation. It was found that, in stable COPD patients exhibiting presence of typical airway bacteria, higher bacterial load correlated with more severe airflow limitation (r=-0.34; p=0.01) (Figure 5.3).

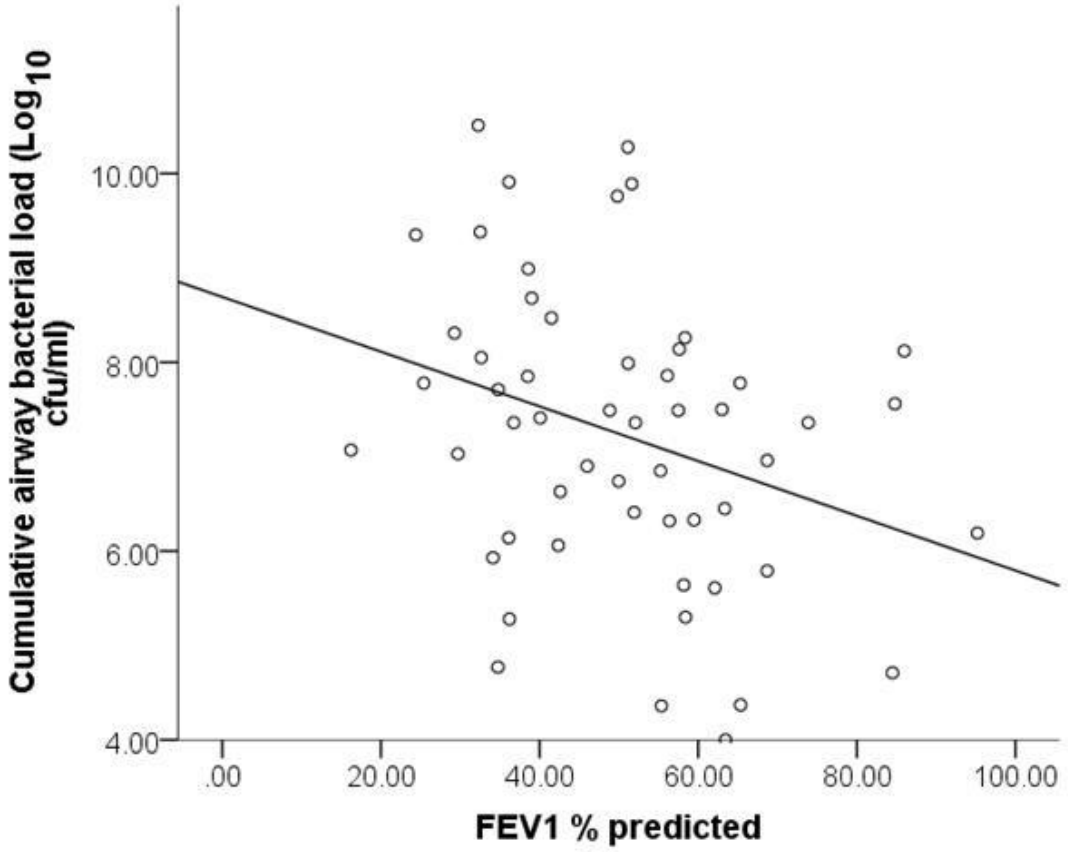


Figure 5.3. Relationship between typical airway bacterial load and airflow limitation (Rho= -0.34; p=0.01).

The patients were sub-divided according to infecting species (Figure 5.4). There was no significant difference in lung function between patients infected with any one species of typical airway bacteria, compared to those with no typical airway bacterial presence. However, it was found that lung function was significantly lower in patients co-infected with at least two types of typical airway bacteria (FEV_1 40.7% predicted) compared with patients without presence (FEV_1 55.4% predicted) (p=0.02).

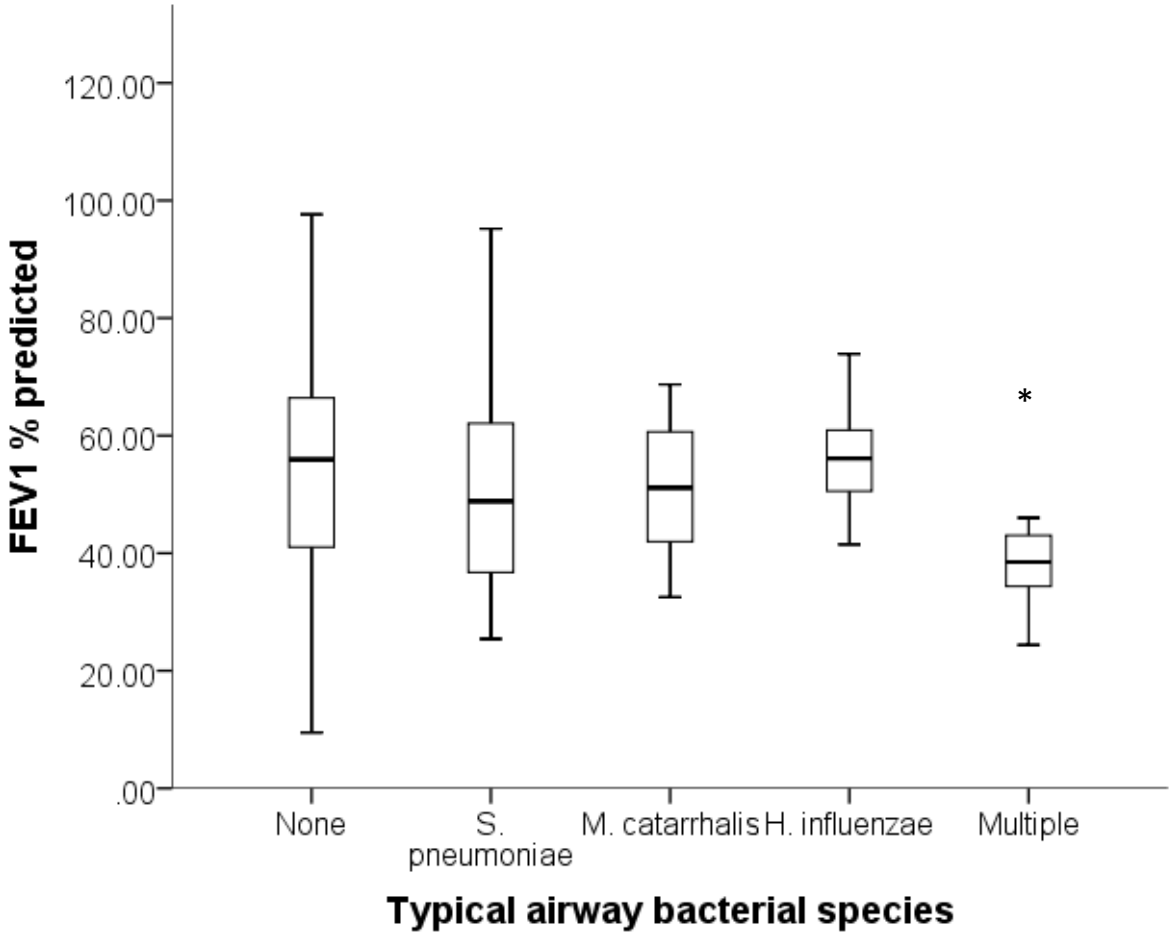


Figure 5.4. Comparison of percentage predicted of forced expiratory volume in 1 second (FEV_1% predicted) dependent on typical airway bacteria presence. The 'Multiple' category relates to samples harbouring >1 type of typical airway bacterial species. *p<0.05 (versus no typical airway bacteria presence).

5.3.5 Systemic inflammation and inhaled corticosteroid therapy

Of the 164 stable state samples with CRP data recorded, ICS data was also available for 152 samples (92.7%). ICS treatment type is illustrated in Table 5.4.

Corticosteroid	% of samples
None	19.7
Fluticasone	57.9
Budesonide	15.1
Beclomethasone	7.2

Table 5.4. Inhaled corticosteroid usage in sampled patients from the London COPD Cohort.

Fluticasone was the most commonly prescribed ICS, being utilised in 88 cases, with budesonide (23 cases) and beclomethasone (11 cases) also occasionally used. Thirty cases (19.7%) did not involve use of ICS therapy at stable state in this dataset. Median (IQR) beclomethasone-equivalent dose levels for the respective ICS therapies are shown below (Table 5.5). Fluticasone prescription involved a significantly higher dose than budesonide and beclomethasone (both $p<0.001$).

Corticosteroid	Median (IQR) beclomethasone-equivalent ICS dose (μg)
Fluticasone	2000 (1000-2000)
Budesonide	640 (320-640)
Beclomethasone	1000 (250-1000)

Table 5.5. Median ICS dose prescribed for each ICS type.

There was no significant difference in median (IQR) levels of systemic inflammation in those patients on maintenance ICS therapy (2 (1-7) mg/L) compared to those not on such therapy (3.5 (2-7) mg/L), p=0.36. Additionally, there was no significant difference in systemic inflammation identified between different treatments of ICS (p=0.51) (Figure 5.5).

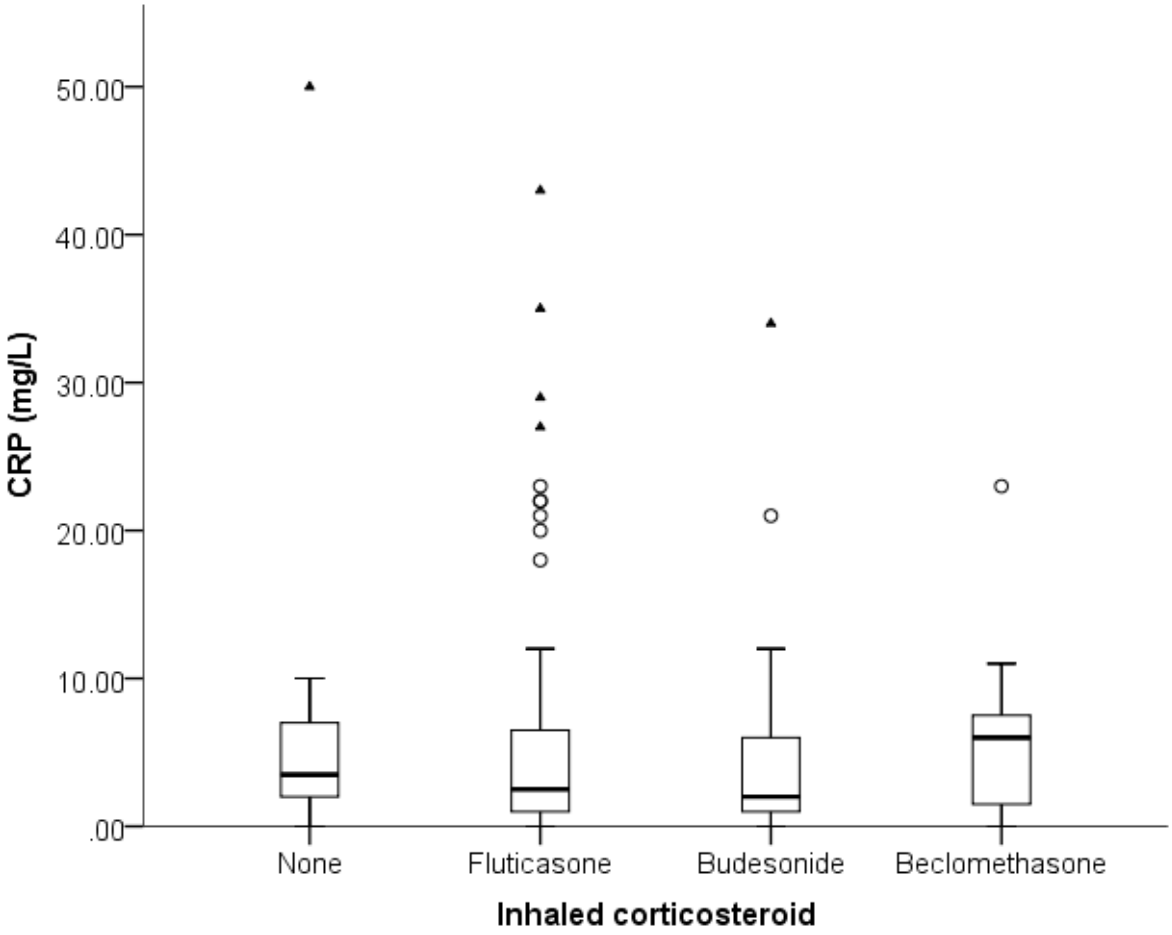


Figure 5.5. CRP levels according to ICS prescription. Outliers shown with a circle are >1.5 times outside the IQR; Outliers shown with a triangle are >3 times outside the IQR.

5.4 Inhaled corticosteroid therapy during the stable state

Given that there was no clinically significant difference in systemic inflammation identified with or without presence of typical airway bacteria, it was necessary to assess the anti-inflammatory regimens of these patients, to determine whether maintenance therapy was playing a major part in controlling such inflammation in patients at the stable state.

In order to assess the correlation between ICS dose and clinical factors in stable COPD, the dosage for each ICS was standardised according to equivalence to beclomethasone. Of the 152 samples which had ICS usage recorded, 30 (19.7%) did not have any ICS usage at the time of sampling.

It was found that patients with stable COPD in whom typical airway bacteria were detected had significantly higher median (IQR) dose of ICS prescribed (Figure 5.6) compared with patients in whom no typical airway bacteria were detected: 2000 (640-2000) µg versus 1000 (320-2000) µg (p=0.018).

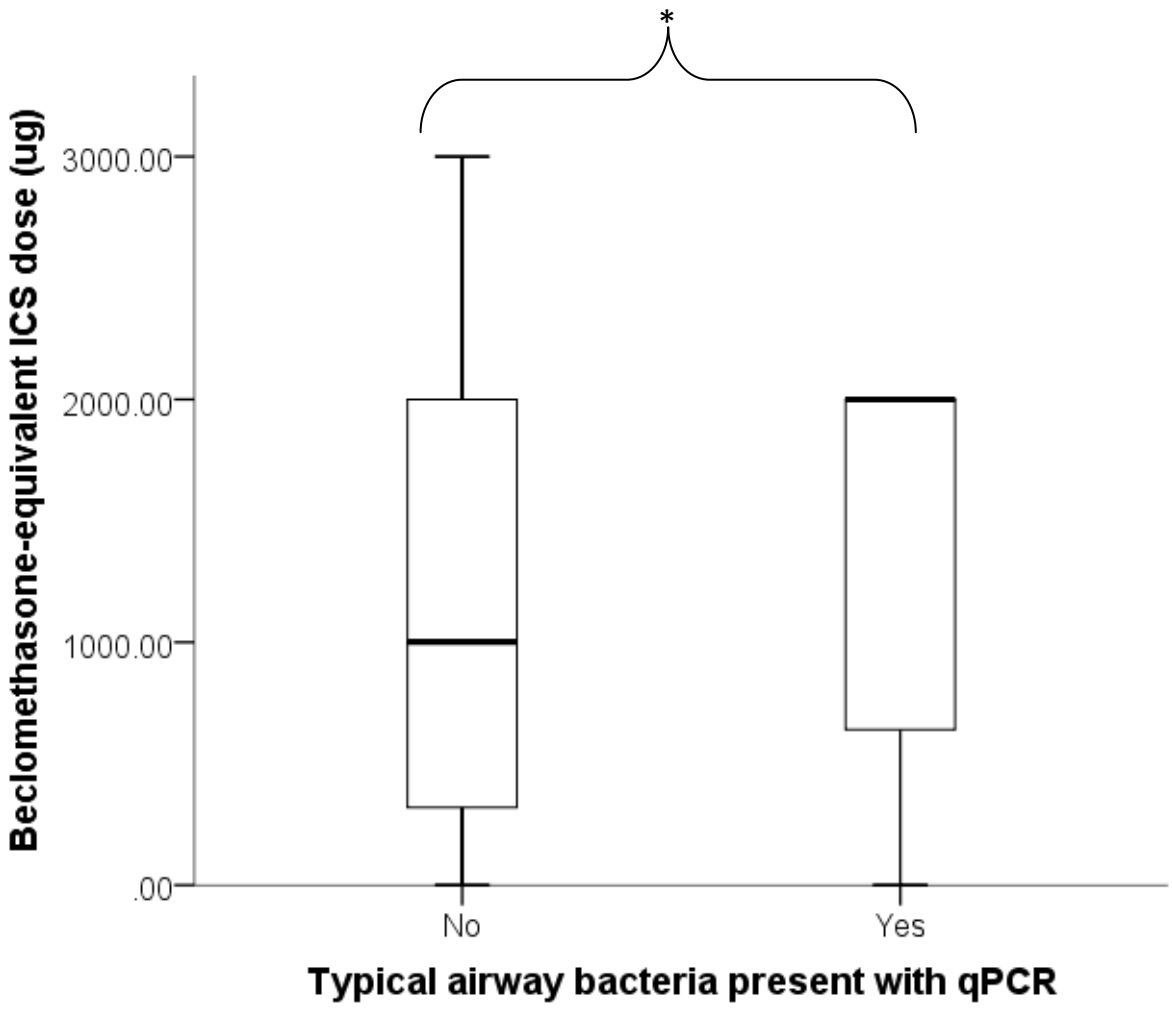


Figure 5.6. ICS dosage prescribed, sub-divided according to typical airway bacterial presence. *p<0.05

There was also a positive correlation between ICS dosage and typical airway bacterial load (rho=0.20; p=0.01). However, there was no correlation between ICS dosage and CRP levels (rho=0.09; p=0.28) (Figure 5.7).

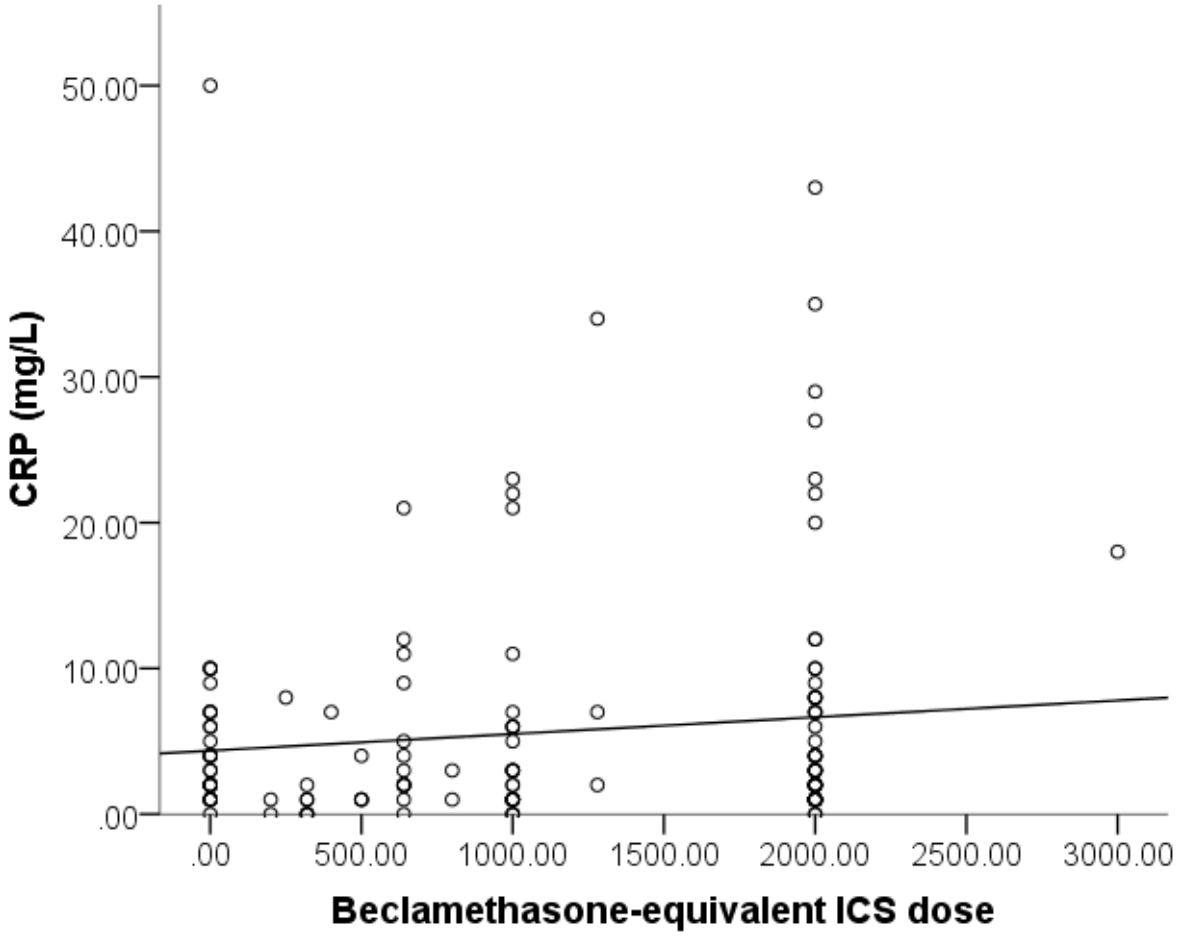


Figure 5.7. Relationship between ICS dosage and CRP in stable COPD patients (n=152) (Rho= 0.09; p=0.28).

In samples positive for typical airway bacteria and in whom ICS usage was measured (n=47), a univariate analysis found that there was a significant correlation between airway bacterial load and ICS dose (rho=0.38; p=0.01) (Figure 5.8). This relationship remains significant in a multivariate analysis which accounts for age, smoking status and FEV_1 % predicted (p=0.022).

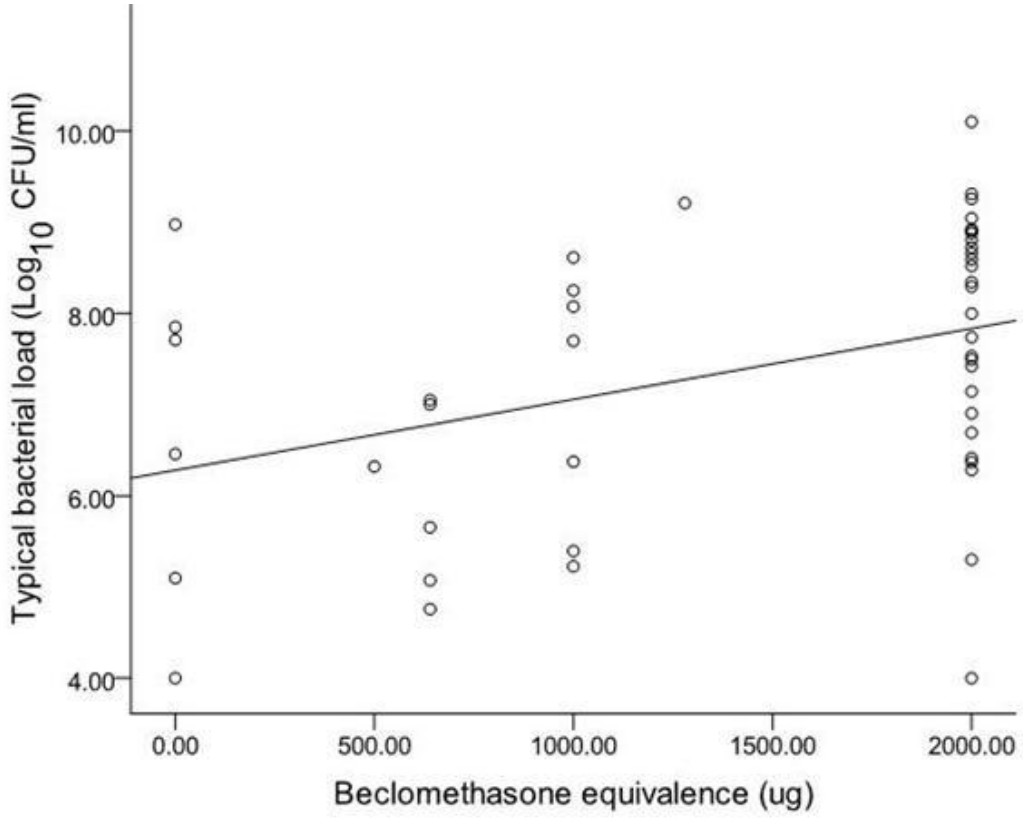


Figure 5.8. Relationship of ICS dosage with airway bacterial load in stable COPD patients (n=47) (Rho= 0.38; p=0.01).

5.4.1 Inhaled corticosteroids and airflow limitation

ICS dosage was compared amongst patients at differing COPD GOLD stages (Figure 5.9). The performance of a Kruskal-Wallis test demonstrated that there was a significantly higher ICS dosage at higher GOLD stages (p=0.049).

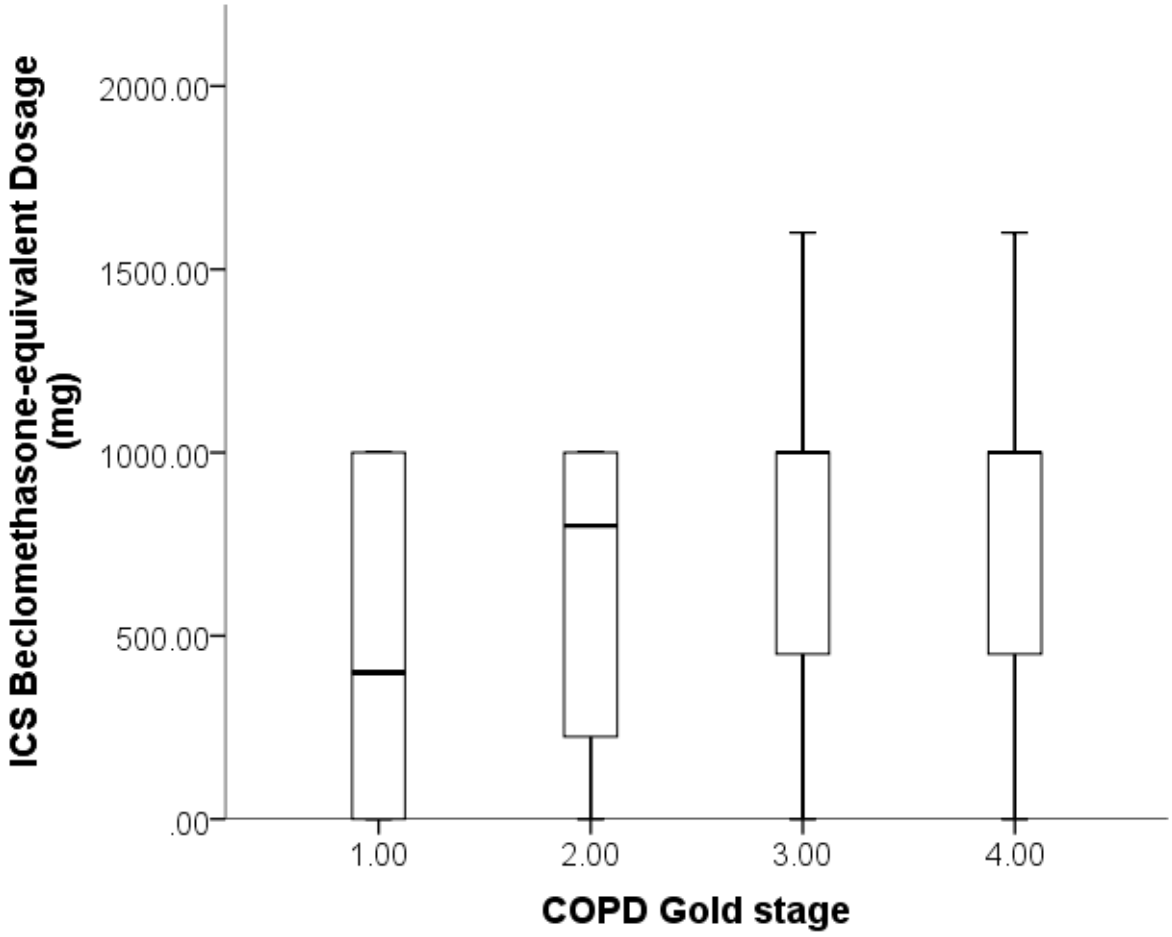


Figure 5.9. Inhaled corticosteroid dosage for stable COPD patients at different GOLD stages.

There was also a correlation between ICS dosage and more severe airflow limitation (rho = -0.27; p=0.001), (Figure 5.10).

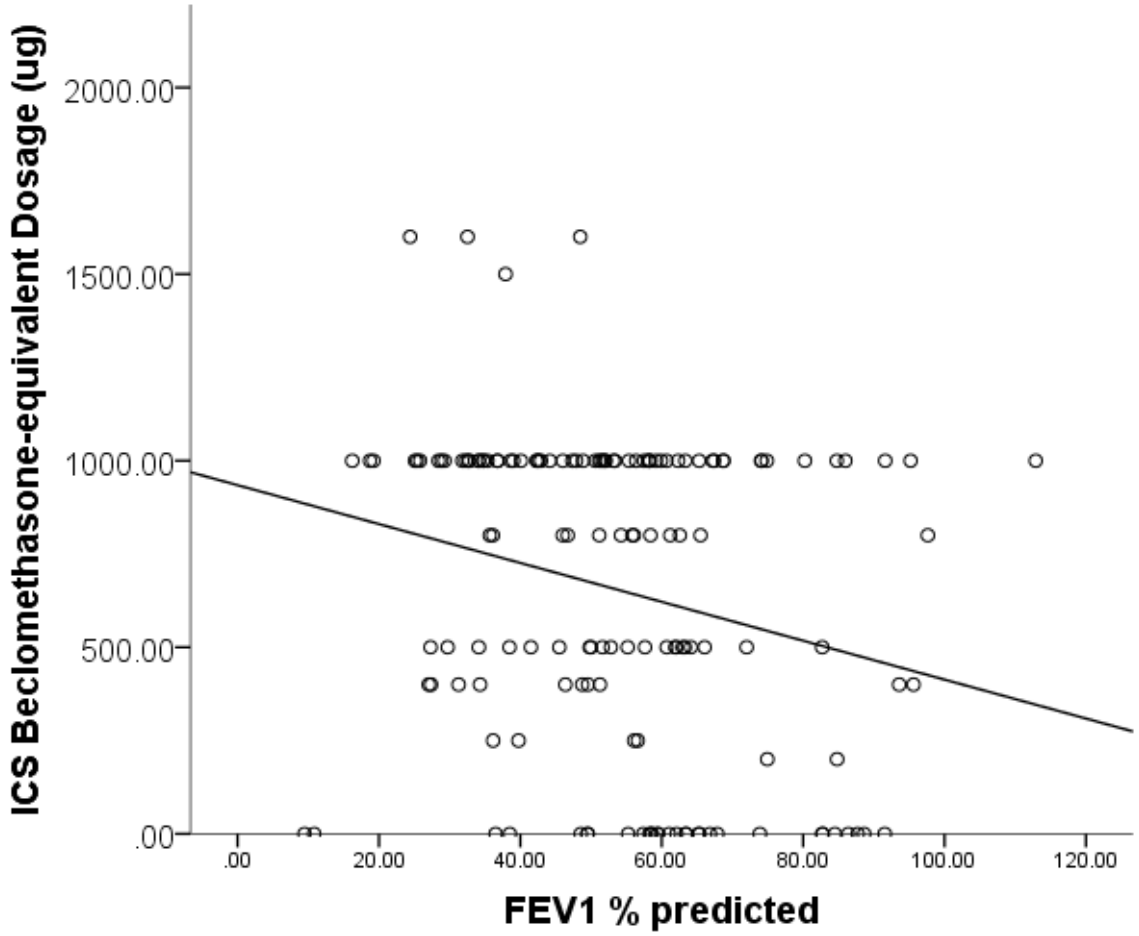


Figure 5.10. Relationship between airflow limitation and ICS dosage (n=149) (Rho= -0.27; p=0.001)

5.5 Discussion

There is a growing body of evidence demonstrating that presence of typical airway bacteria is associated with a more severe inflammatory response in stable COPD (Bresser et al. 2000; Hill et al. 2000; Sethi et al. 2006).

The current chapter further examined this association between microbiological and clinical factors in stable COPD. In particular, the link between typical airway bacterial prevalence and load with systemic inflammation was assessed, and the relationship between typical airway bacteria and prescription of ICS was also explored, with a consideration of how these factors may influence the airflow limitation identified in COPD.

An initial finding of this study was that there was no difference in systemic inflammation between patients with typical airway bacterial presence and those without presence. However, looking specifically at those patients with typical airway bacterial presence, there was found to be a fairly strong positive correlation between typical airway bacterial load and systemic inflammation (Figure 5.1), and although anticipated, to the author's knowledge this is the first time that this relationship has been shown. Additionally, severity of airflow limitation was not found to be related to CRP (Figure 5.2).

It is debated whether bacterial presence is pathogenic or simply a commensal population (Hirschmann 2000; Murphy et al. 2000; Murphy 2006). The evidence

from the current study indicates that whilst bacterial presence may not be pathogenic at low load, it is having a pro-inflammatory effect at higher loads. Indeed, at the stable state it was identified that bacterial presence is associated with a median CRP value of 2 mg/L, whilst bacterial infection is typically associated with a CRP value of >40 mg/L (Clyne & Olshaker 1999). This suggests that either the typical airway bacterial presence seen is not sufficient to enact the sequelae of an acute bacterial infection (i.e. it is unable to initiate an exacerbation), or that ICS prescription is inhibiting the increase in systemic inflammation, or both.

It was found that systemic inflammation, as measured by CRP, was positively correlated to the typical airway bacterial load in those stable COPD patients who had presence of at least one typical airway bacterial species (rho=0.30).

Typical airway bacterial species are known to contribute to both local and systemic inflammation as part of their pathology during acute respiratory infections (Murphy 2006; Yu et al. 2010) as well as during chronic disease (Hurst et al. 2005; Hill et al. 2000). Therefore, the positive correlation between CRP and bacterial load identified in the current investigation provides evidence that even during the stable state, this load is contributing to a pro-inflammatory response. These findings have potentially profound implications for the treatment of COPD in the stable state, including the importance in considering the impact of prophylactic antibiotic therapy for patients with COPD, during the stable state, as this may reduce bacterial load in such patients. Two recent trials have investigated the use of prophylactic therapy using macrolide antibiotics. In 2008, Seemungal and colleagues published

data on the London COPD Cohort demonstrating that prophylactic treatment for one year with the macrolide antibiotic erythromycin led to a significant reduction in exacerbations compared with placebo, in a total of 109 outpatients (53 on macrolides, 56 on placebo) (Seemungal et al. 2008). The authors did not find differences between macrolide and placebo groups in levels of airway and systemic inflammatory markers. However, in that study, *H. influenzae* (in 27% of stable samples) was considered resistant to erythromycin, and this may have prevented bacterial load from being reduced. A later trial by Albert and colleagues investigated azithromycin as the macrolide antibiotic, with a total of 1142 patients (570 on azithromycin, 572 on placebo) (Albert et al. 2011). They also identified that macrolide treatment reduced exacerbation frequency. They did not, however, examine airway or systemic inflammatory markers in these patients. It is important to note that the mechanism of how the antibiotic works in COPD has not yet been elucidated, as macrolides have both antibiotic and anti-inflammatory effects. The current study suggests that prophylactic antibiotic treatment, using antibiotics to which typical airway bacteria are sensitive, would lower bacterial load in stable COPD patients and this would directly lead to lower systemic inflammatory markers.

The current study has, for the first time identified a correlation between airway bacterial load and ICS dosage in stable patients at the time of sampling (Figure 5.7), independent of age, smoking status and disease severity. Previous findings from the TORCH and INSPIRE randomised clinical trials have suggested that ICS use is related to a higher frequency of pneumonia development in COPD patients

(Calverley et al. 2007; Calverley et al. 2011; Wedzicha et al. 2008). In TORCH it was shown that in patients treated with salmeterol/fluticasone combination ICS, the rate of pneumoniae was 88 per 1000 treatment-years, compared with 52 per 1000 treatment-years on placebo, an increase of 69%. The current study shows that higher ICS dose is associated with higher airway bacterial load and this may play a part in increasing susceptibility to pneumonia in COPD.

This study has considered the interlinked factors of typical airway bacteria presence, systemic inflammation and anti-inflammatory therapy in the disease outcome during stable COPD. It has established that bacterial load is associated with higher levels of CRP, more severe airflow limitation, and also that co-infection with more than one typical airway bacteria specifically is associated with poorer lung function. It has also identified that prescription of higher inhaled corticosteroid dosage is seen in patients with higher bacterial loads.

CHAPTER 6. **Changes in Airway Bacteria Prevalence and Load at Exacerbation and during Exacerbation Recovery**

6.1 Introduction

It has been demonstrated that prevalence and load of typical airway bacteria increases from stable state to exacerbation, as highlighted in Chapter 4 of this thesis, building upon previous analyses in the literature using culture (Rosell et al. 2005; Monso et al. 1995). The changes that occur in bacterial prevalence and load during the recovery period following an exacerbation have not previously been defined in the literature, despite the use of antibiotic therapy in this period. The role of antibiotic therapy during an exacerbation is controversial (Hirschmann 2000), and this has previously been considered in chapter 5 of the current study.

Historically, only small improvements were seen in trials comparing antibiotic versus non-antibiotic treatments for COPD exacerbations, in favour of treatment (Saint et al. 1995). Saint and colleagues performed a meta-analysis on 9 trials, performed between 1957 and 1992, showing an overall summary effect size of 0.22 in the antibiotic group compared to the placebo group, with 7 of the 9 trials analysed favouring antibiotic use. A recent study by Llor and colleagues demonstrated that antibiotic therapy in mild-to-moderate exacerbations is both more effective at clinical cure within 9-11 days (74.1% vs 59.9%) and significantly lengthens time to next exacerbation (233 days vs 160 days) compared to placebo (Llor et al. 2012). In addition, a retrospective study of almost 85,000 COPD patients investigating more severe exacerbations, resulting in hospitalisation, also demonstrated that patients treated with antibiotics had lower risk (1.1% vs 1.8%) of treatment failure (the necessity of ventilation treatment after the second hospital

day), lower inpatient mortality rates (1.0% vs 1.6%), and lower readmission rates (within 30 days of index exacerbation) for exacerbation events (7.91% vs 8.79%) compared to no treatment (Rothberg et al. 2010).

Roede and colleagues examined the effect of corticosteroid treatment with or without antibiotic treatment following an exacerbation (Roede et al. 2009). They found that time to next exacerbation was lengthened for those patients treated with antibiotics (258 days in antibiotic group versus 189 days in non-antibiotic group). Each of these studies implicitly suggest that the impact of antibiotics on the microbial population is a positive factor in such clinical outcomes. However, as of yet, no investigation of the mechanistic rationale for these changes have taken place. During the stable state, it has also previously been demonstrated that antibiotic therapy for 5 days significantly reduces bacterial prevalence at two-weeks post-treatment start (Miravitlles et al. 2009). Miravitlles and colleagues found that moxifloxacin therapy was associated with the elimination of 'potentially pathogenic microorganisms' in 75% of patients, with placebo treatment associated with elimination in 30% of patients. At the 8-week follow up, however, airway bacterial prevalence was similar in both groups (80% in placebo group, 75% in moxifloxacin group.

Recurrent exacerbations are of major concern in COPD. It has previously been identified that approximately 22% of patients suffer a recurrent exacerbation within 50 days of an index exacerbation (Perera et al. 2007). This indicates a major burden in terms of morbidity and mortality. Perera and colleagues highlighted that the

presence of systemic inflammation, as measured by the biomarker C-reactive protein (CRP), is associated with such recurring events. Serum CRP is also known to be related to presence of bacterial infections in COPD exacerbations (Peng et al. 2013).

COPD exacerbations are frequently treated with a course of antibiotics and systemic corticosteroids following diagnosis (Wenzel et al. 2012; Walters et al. 2011), as discussed in section 1.2.1.

The airways microbiome refers to the total bacterial population in the airways. This microbiome has been explored in recent years following the utilisation of molecular techniques such as restriction fragment length polymorphism, microarray analysis and sequencing (Hilty et al. 2010; Huang et al. 2010; Sze et al. 2012). However, bacterial load has been little-studied, and in previous studies has involved very small numbers of subjects (median 8 subjects per sample group), leading to limitations in the interpretation of results (Sze et al. 2012). The load of the airway microbiome at exacerbation presentation and its relationship to exacerbation severity is as yet unknown, and additionally, the impact of antibiotic therapy on the microbiome burden in the post-exacerbation recovery phase has not been analysed.

According to the most recent GOLD COPD guidelines, antibiotics can shorten recovery time, and improve lung function (FEV_1), as well as reducing the risk of a relapse and length of hospital stay (www.goldcopd.org). A systematic review of

antibiotics during acute exacerbations of COPD demonstrated that antibiotic usage decreased in-hospital mortality by 78% (Quon et al. 2008).

In order to study bacteriological changes during exacerbations, we investigated prevalence and load of *H. influenzae*, *S. pneumoniae* and *M. catarrhalis*, and load of the total airway bacterial population, during exacerbation recovery using qPCR.

6.2 Patient characteristics

We collected sputum from 94 subjects in the London COPD cohort at exacerbation presentation (n=135), day 3 (n=41), day 7 (n=53), day 14 (n=53) and day 35 post-exacerbation (n=38). Patient characteristics for these 94 patients is illustrated in Table 6.1

Characteristic	
Median age (range), years	70.4 (47.4-87.5)
Male gender (%)	60.6
Current smoker (%)	22.3
Median pack-year smoking history (IQR)	44.5 (27.5-63.2)
Mean FEV1 (SD), Litres	1.14 (0.5)
FEV1 % predicted (SD)	46.2 (17.7)
FEV1/FVC ratio (SD)	0.44 (0.1)
Mean FVC (SD), Litres	2.6 (0.9)

Table 6.1. Baseline characteristics of 94 patients of the London COPD Cohort, who participated in a study of typical airway bacterial presence during acute exacerbations of COPD. No significant differences are seen between these and the patients described in Table 3.5.

6.3 Results

6.3.1 Antibiotic treatment type and duration

Of the 135 exacerbations, 126 (93%) were treated with antibiotics, prescribed at exacerbation presentation. Of the 9 patients not prescribed antibiotics, two (22.2%) had typical airway bacteria (*H. influenzae*, *S. pneumoniae* or *M. catarrhalis*) detected at exacerbation. The type of antibiotic course prescribed was based on clinical judgment of symptoms and patient history, and follows GOLD COPD guidelines (www.goldcopd.org). Median antibiotic course length was seven days. Antibiotics prescribed are shown in Figure 6.1.

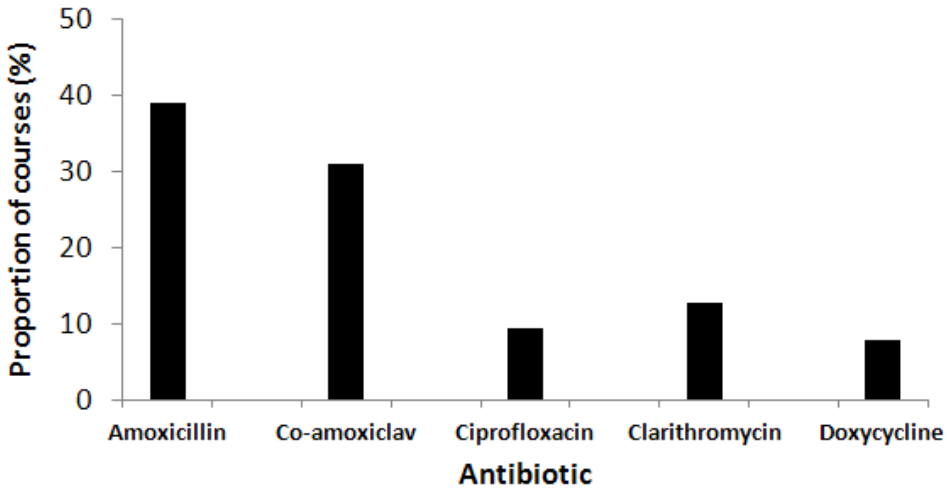


Figure 6.1. Antibiotics prescribed to patients in this study (n=126). A further 9 patients had no antibiotics prescribed at exacerbation visit.

6.3.2 Prevalence and load of typical airway bacteria during exacerbation recovery

The prevalence of typical airway bacteria was found to be significantly greater at exacerbation presentation compared with the following two weeks of exacerbation recovery: versus Day 3 (50.4% vs 26.8%; p=0.008), day 7 (28.3%; p=0.006) and day 14 (32.1%; p=0.02), whilst the difference in prevalence versus day 35 (39.5%) was not significant (p=0.21) (Figure 6.2). When recurrent exacerbations were excluded from the analysis, the prevalence of typical airway bacteria was 51.5% at exacerbation (n=101) and 35.3% at day 35 (n=34), although this difference was still not significant (p=0.10)

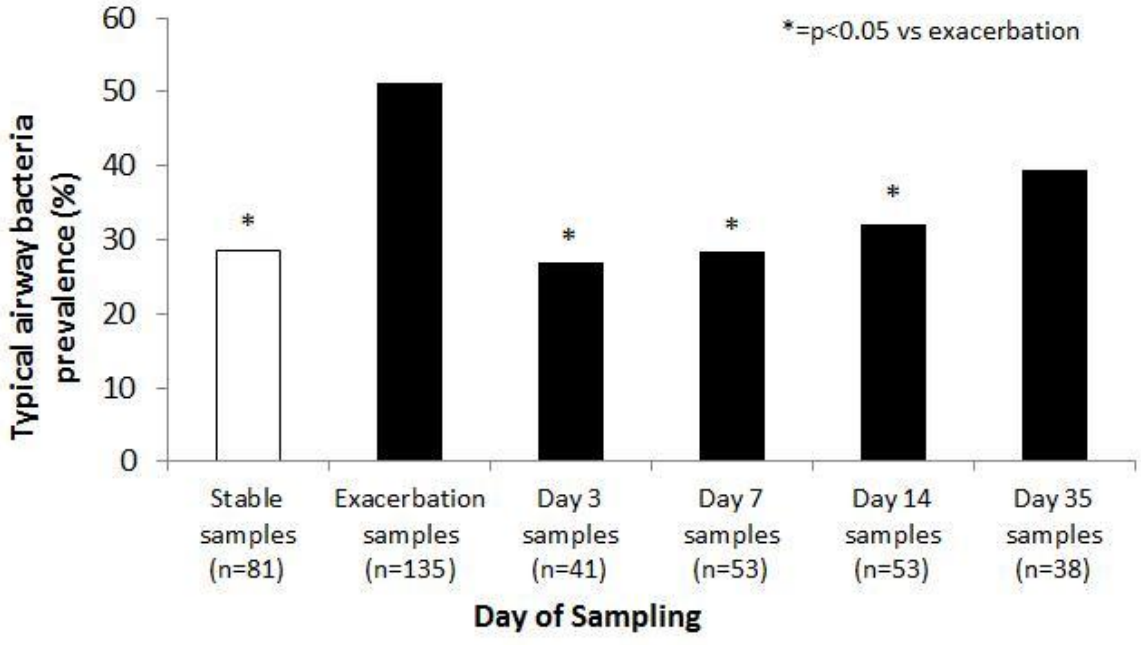


Figure 6.2. Bacterial prevalence at stable, exacerbation and recovery phases of COPD. Bacterial prevalence is highest at exacerbation presentation, declining significantly by three days post-presentation. However, prevalence increases by day 35 post-presentation.

Typical airway bacterial load was examined using both longitudinal and cross-sectional analyses. In the longitudinal analysis, those patients who had a typical bacteria-positive exacerbation had this exacerbation sample paired with their samples at the respective recovery time points: load [median (IQR) Log_{10} CFU/ml] was significantly higher in patients at exacerbation visit than at day 3 (n=15): $10^{8.1(5.9\text{-}8.7)}$ vs $10^{0(0\text{-}4)}$, p=0.001; day 7 (n=17): $10^{8.2(6.3\text{-}8.8)}$ vs $10^{4.2(0\text{-}6.6)}$, p=0.01; day 14 (n=14): $10^{8.0(6.3\text{-}8.7)}$ vs $10^{4.4(0\text{-}6.6)}$, p=0.022; and day 35 (n=15): $10^{7.7(5.7\text{-}9.3)}$ vs $10^{5.9(0\text{-}6.74)}$, p=0.047 (Figure 6.3).

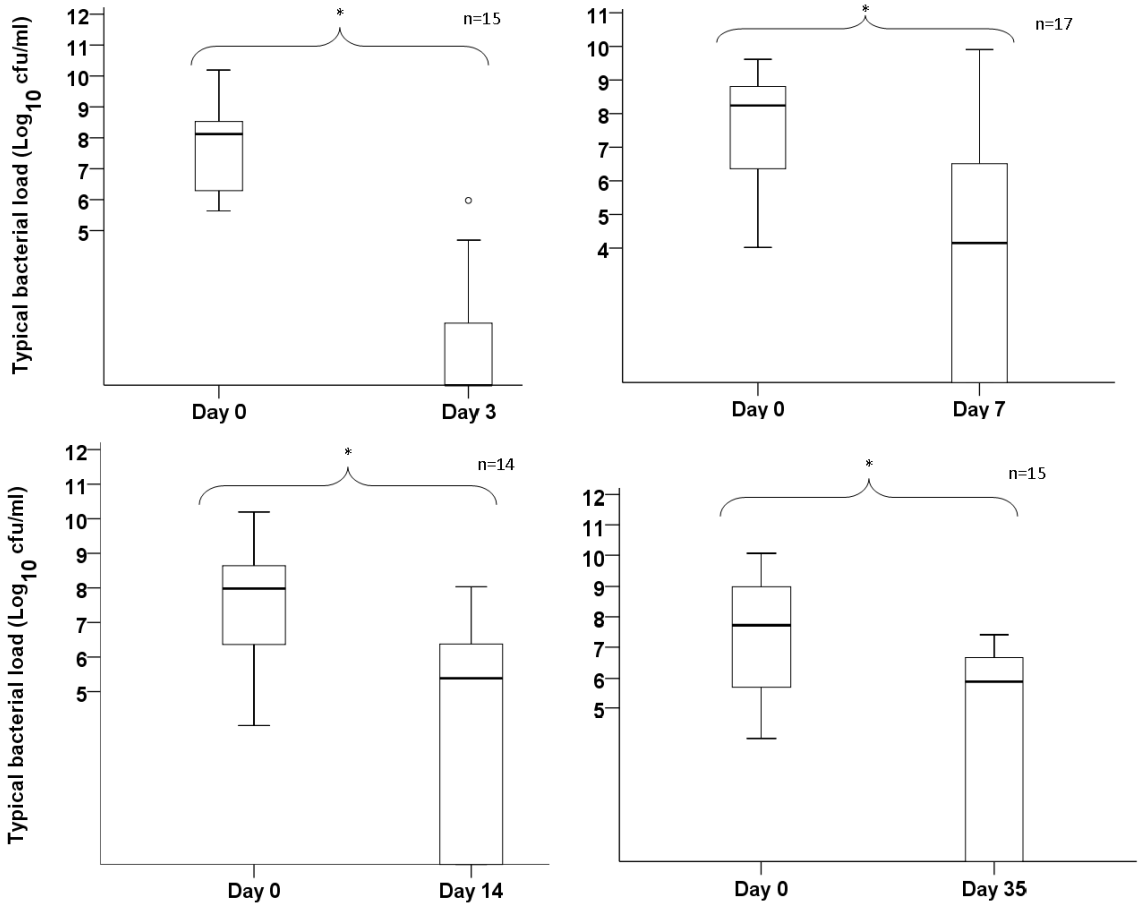


Figure 6.3. Typical airway bacterial load changes in paired exacerbation and recovery samples. Each boxplot illustrates paired load analysis between the load seen at exacerbation presentation and the load seen at the respective timepoint (day 3, 7, 14, 35). All episodes were positive for at least one typical airway bacterial species at exacerbation presentation.

In the cross-sectional analysis, samples positive for at least one typical airway bacterial species at the respective time points were assessed. Examining only these samples, bacterial load was found to be significantly higher at exacerbation compared to Day 3 ($10^{8.0(\pm0.2)}$ vs $10^{6.3(\pm0.5)}$ CFU/ml; p<0.001), Day 7 ($10^{8.0(\pm0.2)}$ vs $10^{7.0(\pm0.4)}$; p=0.012), Day 14 ($10^{8.0(\pm0.2)}$ vs $10^{7.0(\pm0.3)}$; p=0.005) and Day 35 ($10^{8.0(\pm0.2)}$ vs $10^{6.9(\pm0.3)}$; p=0.004) (Figure 6.4), echoing the findings of the longitudinal analysis.

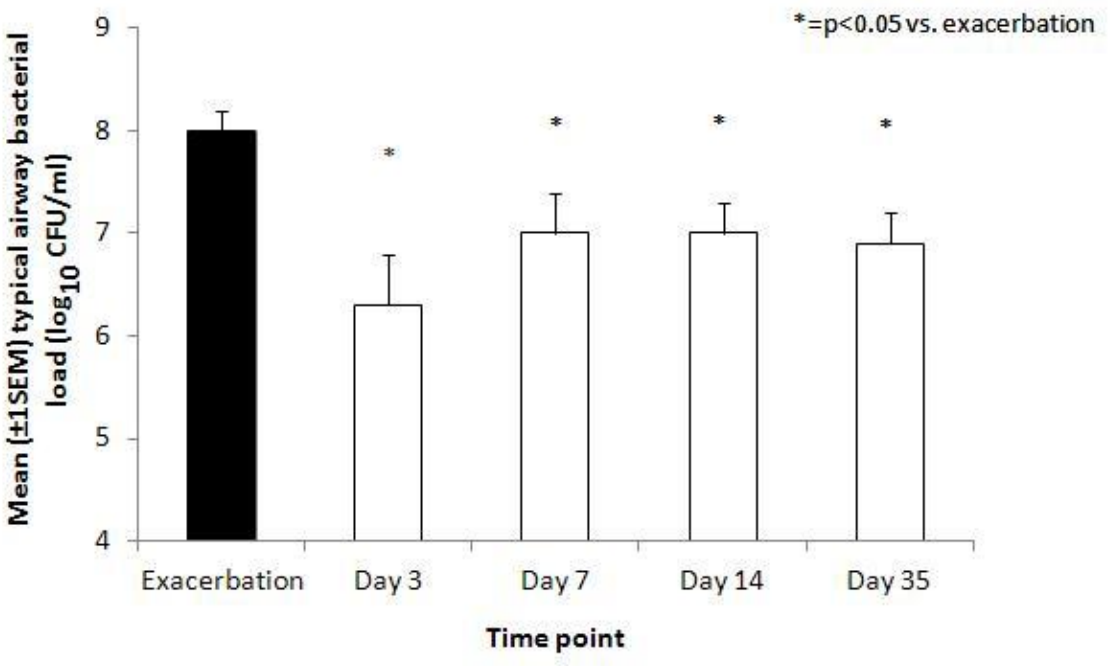


Figure 6.4. Cross-sectional analysis demonstrating differences in typical airway bacterial load at the respective recovery time points (n-value for each time point: Exacerbation (n=69), Day 3 (n=11), day 7 (n=15), day 14 (n=17) and day 35 (n=15)).

6.3.3 Prevalence and load of airway microbiome during exacerbation recovery

In a subset of the 320 sputum samples examined for typical airway bacteria, total load of the airway microbiome was also quantified by 16S rDNA qPCR, as described previously in section 2.9 (n=190). It was found that all samples had presence of a microbial population in the airways.

A longitudinal analysis was performed to compare load at exacerbation and follow-up time points: The microbiome burden was significantly higher at exacerbation presentation compared to Day 3 ($10^{9.1(\pm0.1)}$ vs $10^{8.2(0.3)}$; p=0.02) and Day 35 ($10^{9.1(\pm0.3)}$ vs $10^{7.6(\pm0.7)}$, p=0.02) (Figure 6.5).

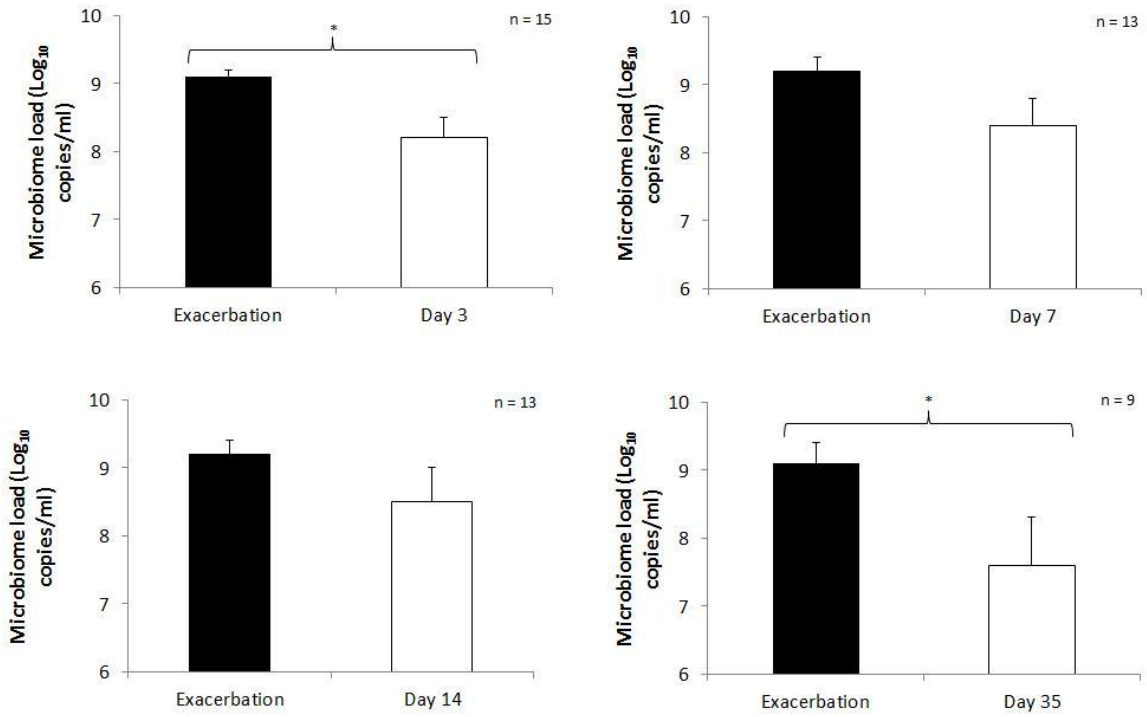


Figure 6.5. Airway microbiome load changes in paired exacerbation (black bars) and recovery (white bars) samples. *p<0.05

6.3.4 Typical airway bacterial load relationship to total microbiome load

Total load of the airway microbiome was also quantified by 16S rDNA qPCR. It was found that total microbiome load was significantly higher in samples where typical airway bacteria were present, compared to those samples where no typical airway bacteria were detected (median $10^{9.7(1.5)}$ vs $10^{8.9(1.3)}$ copies/ml, respectively; $p<0.001$), constituting a six-fold higher median microbiome population in patients with typical airway bacteria than in those without (Figure 6.6).

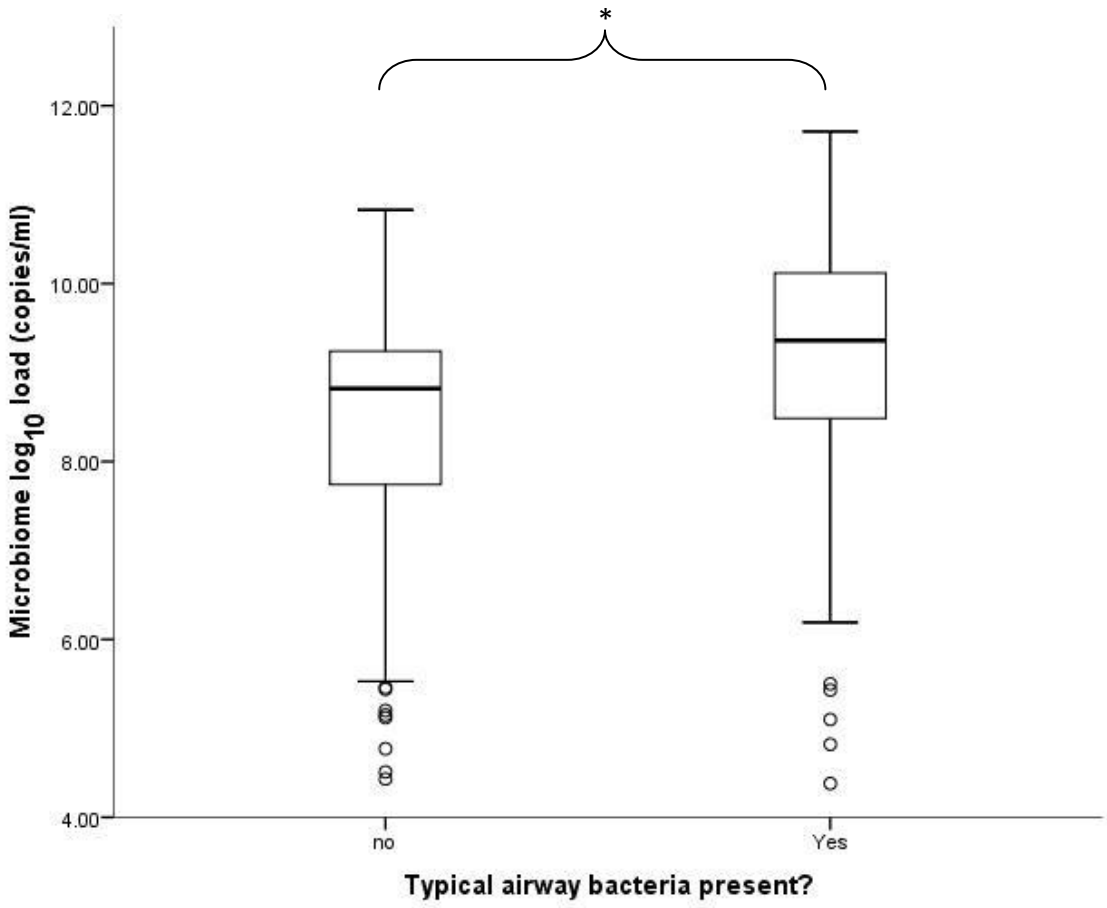


Figure 6.6. Microbiome population size in presence or absence of typical airway bacteria. Outliers shown with a circle are >1.5 times outside the IQR *p<0.05

In addition microbiome load was positively correlated to typical airway bacterial load in those samples which had presence of at least one typical bacterial species (rho=0.41; p<0.001) – for the purpose of direct comparison with microbiome load, the typical airway bacterial load was calculated in copies/ml (Figure 6.7).

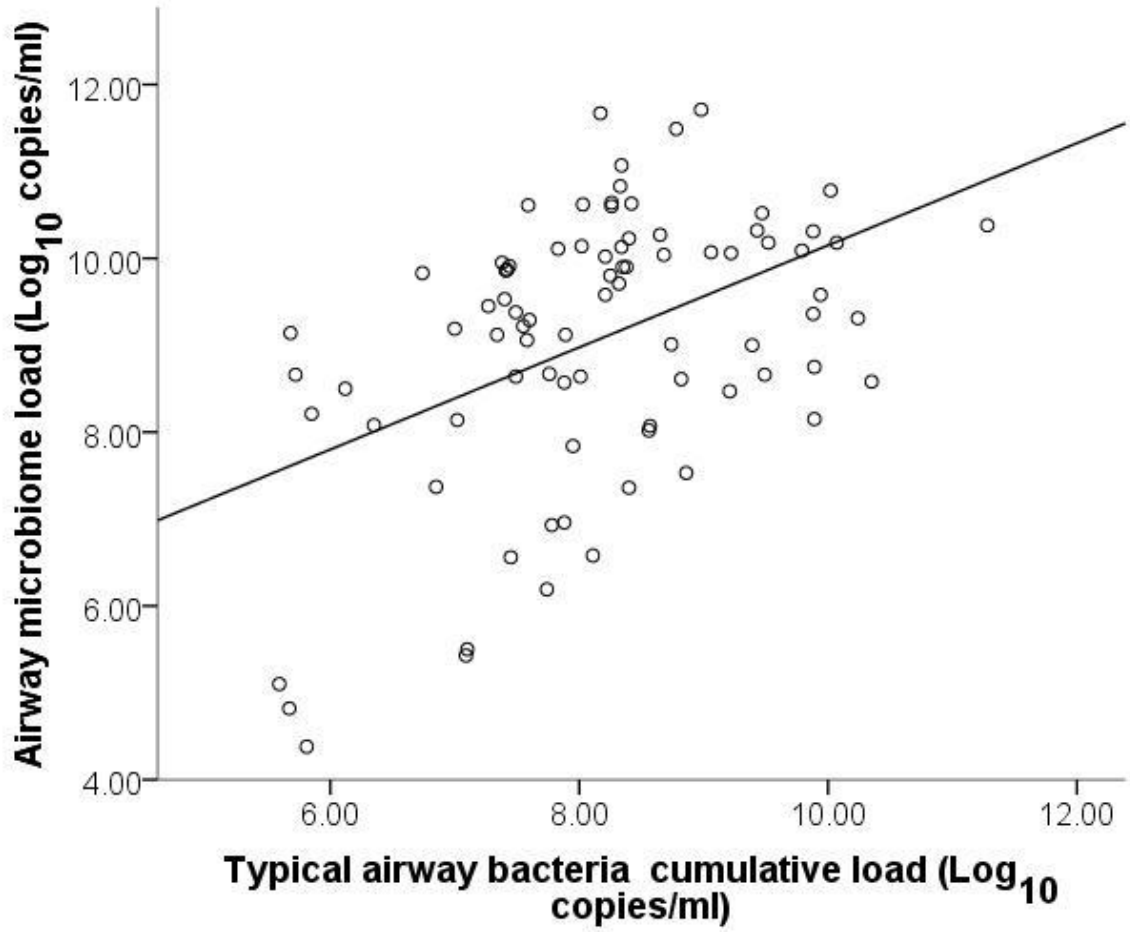


Figure 6.7. Association of microbiome load with typical airway bacterial load during COPD exacerbation and recovery (n=83). This illustrates that a rise in typical airway bacterial load is positively correlated to a rise in load of the overall microbiome population (rho = 0.41; p<0.001)

6.3.5 Systemic inflammation during exacerbation and recovery

In order to establish whether changes in typical airway bacterial load were related to changes in systemic inflammation, total typical airway bacterial load was compared against levels of CRP, a marker of systemic inflammation. In total, 299 samples had typical bacterial load and CRP data provided at exacerbation and recovery. It was found that bacterial load is significantly correlated to CRP ($r=0.25$; $p<0.001$) (Figure 6.8). This correlation is also seen when only those samples positive for typical airway bacteria and with CRP data available (n=119) are assessed ($r=0.28$; $p=0.002$).

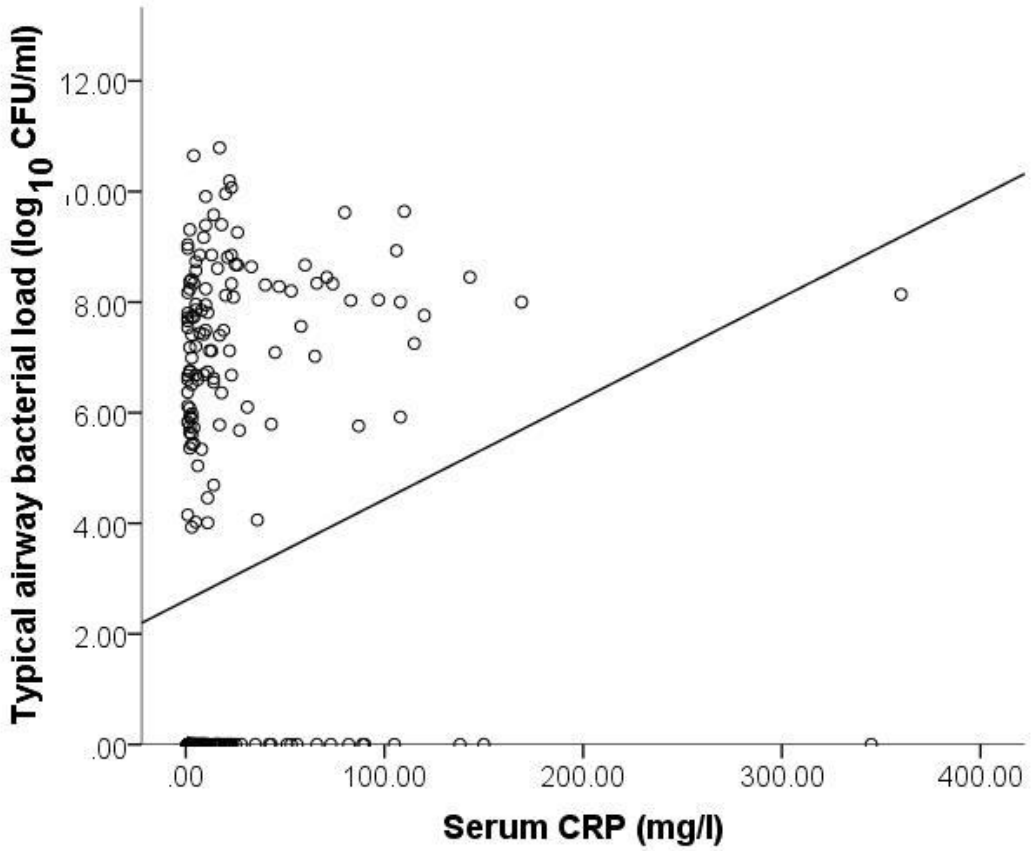


Figure 6.8. Relationship between typical airway bacterial load and systemic inflammation (n=299). Higher load is associated with higher inflammation (rho = 0.25; p<0.001). This significance remains even with removal of outlier with CRP 360mg/l and bacterial load of $10^{8.14}$ CFU/ml (rho = 0.245; p<0.001)

Exacerbations associated with typical airway bacteria had significantly higher median (IQR) serum CRP compared with exacerbations not associated with those bacteria (20 (7.25-52.75) mg/L vs 9.5 (3.25-22) mg/L; p=0.004) (Figure 6.9).

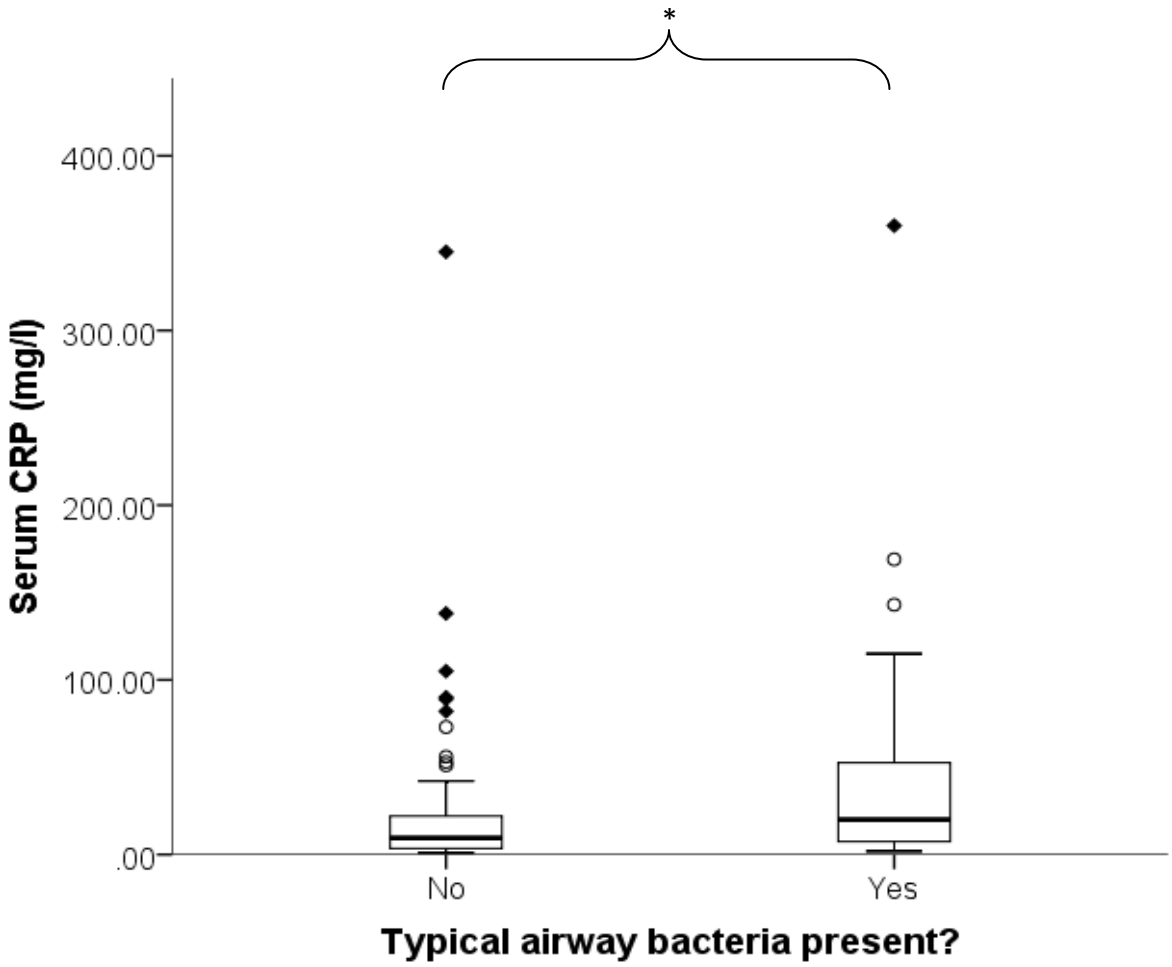


Figure 6.9. Difference in rate of systemic inflammation based on presence or absence of typical airway bacteria. Outliers shown with a circle are >1.5 times outside the IQR; Outliers shown with a diamond are >3 times outside the IQR. *p<0.05

Each of the three typical airway bacteria were then studied in turn to determine if all showed a similar inflammatory response. It was found that *H. influenzae* load was significantly related to CRP (r=0.21; p<0.001). However neither *S. pneumoniae* (r=0.046; p=0.43) nor *M.* catarrhalis (r=0.097; p=0.09) showed a significant relationship to an inflammatory response.

No significant correlation was identified between load of the airway microbiome and systemic inflammation (r=0.021; p=0.77) (Figure 6.10).

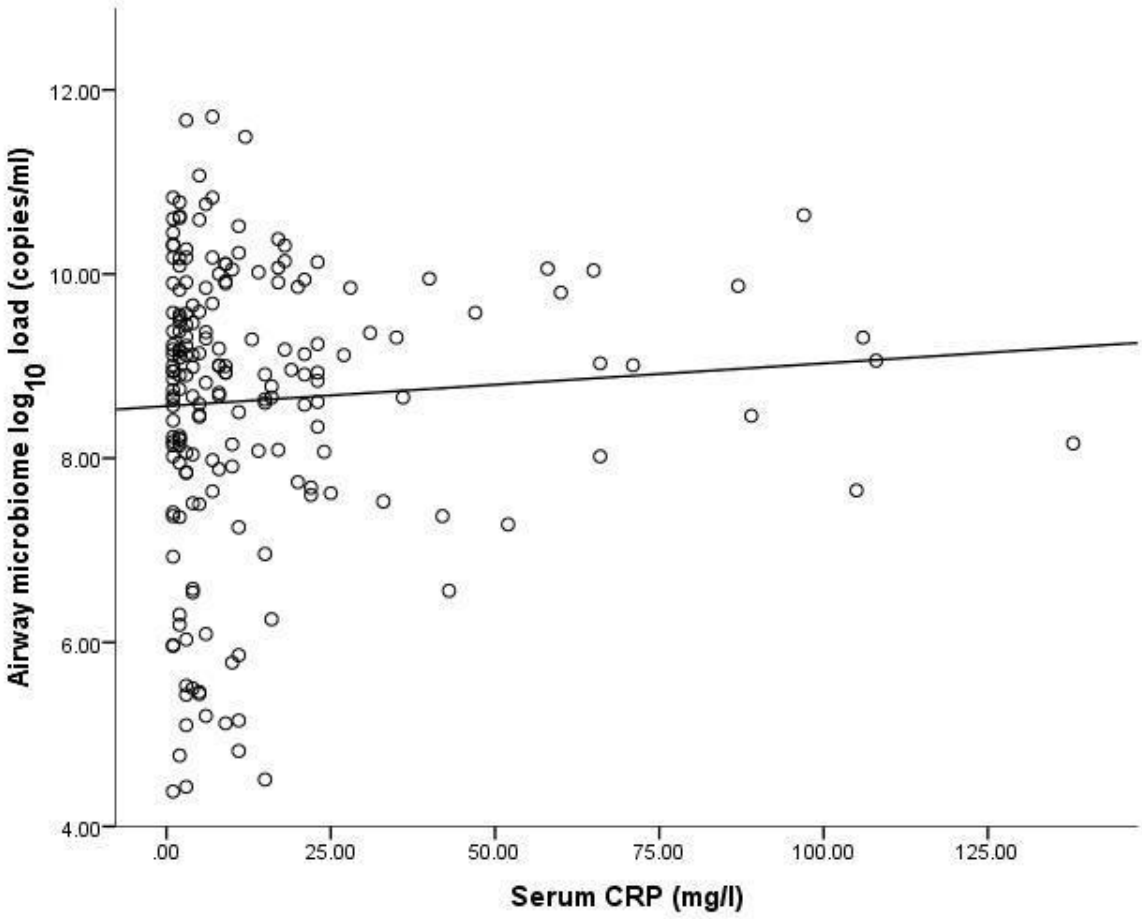


Figure 6.10. Airway microbiome load is not related to systemic inflammation (n=190) (rho = 0.021; p=0.77)

Further analysis elucidated that systemic inflammation, as measured by CRP, is highest at exacerbation, before falling significantly by day 3 post exacerbation visit, and remaining low 35 days post-exacerbation (Table 6.2).

number of samples	Sample Type	Median CRP (IQR) mg/L	CRP change from exacerbation
123	Exacerbation	14 (5-31)	N/A
40	Day 3	4.5 (2-9)	-9.5 mg/L (p<0.001)
51	Day 7	3 (1-6)	-11 mg/L (p<0.001)
48	Day 14	8 (3-17)	-6 mg/L (p=0.005)
37	Day 35	3 (2-8.5)	-11 mg/L (p<0.001)

Table 6.2. Median levels of C-reactive protein at different phases of exacerbation recovery in an unpaired analysis (n=299).

Additionally, in samples for which CRP and spirometry data was available, there was no association between airflow limitation and systemic inflammation at exacerbation presentation (r= -0.11; p=0.23).

6.3.6 Presence of typical airway bacteria during exacerbation recovery does not suggest increased risk of recurrent exacerbation

Recurrent exacerbations - either reported to Cohort clinicians or unreported (recorded in diary cards) - were found to occur following 34/135 index exacerbations (25.2%), within 50 days of the index exacerbation. Presence of typical airway bacteria was not seen to be related to the induction of a recurrent exacerbation at any time point, although the recurrent-exacerbation subset of samples may not have been sufficiently powered to identify variance at each and every time point. Furthermore, there was no significant difference in mean (SEM) airway microbiome load in patients with recurrence and those without ($10^{8.9(\pm0.3)}$ vs $10^{8.8(\pm0.2)}$ copies/ml; p=0.73).

6.3.7 Lung function during exacerbation recovery

It was found that there was no significant difference in forced expiratory volume (FEV_1% predicted) in patients at different stages of exacerbation recovery (p=0.33) (Figure 6.11).

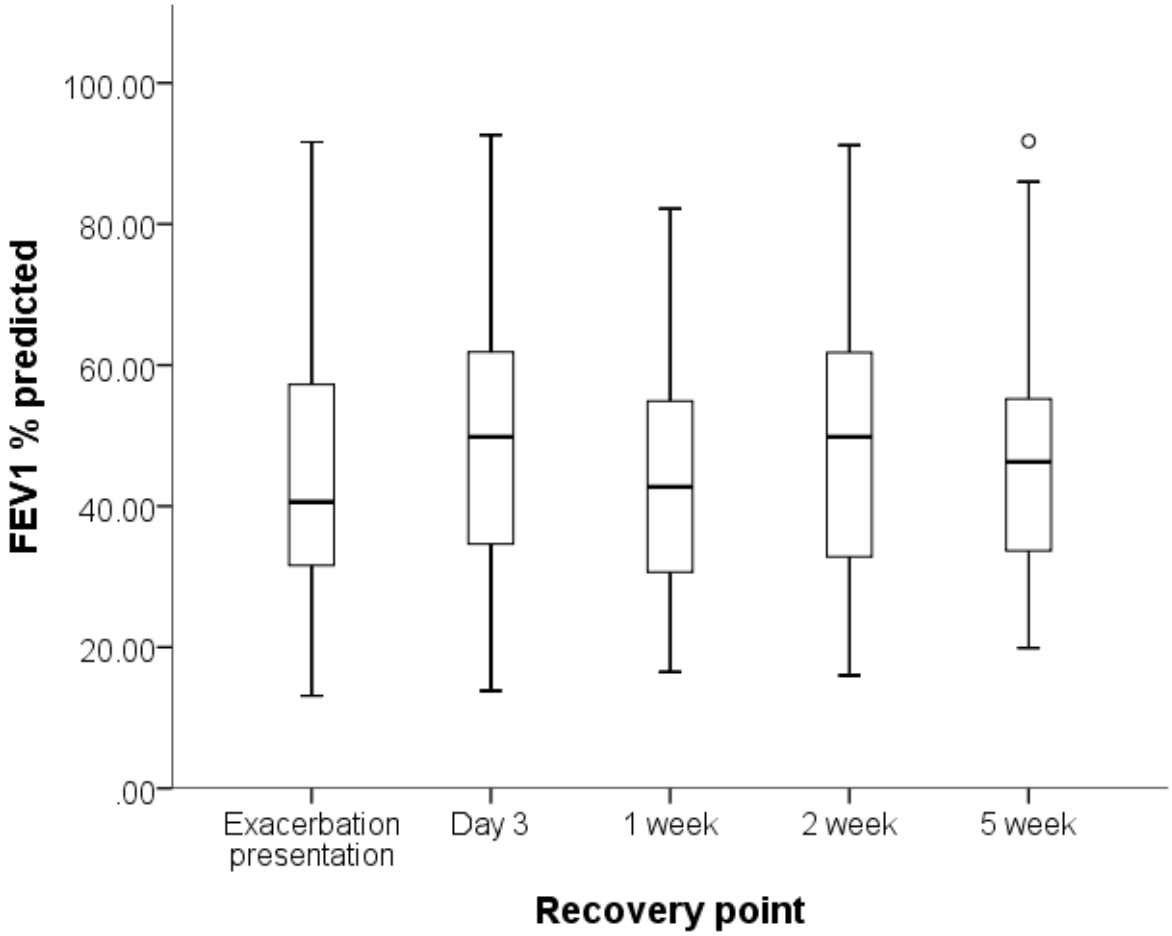


Figure 6.11 . Median FEV_1%predicted does not significantly differ during exacerbation recovery.

There was no significant difference in median (IQR) airflow limitation between samples with or without typical airway bacterial presence (FEV_1% predicted 42.0% (31.5-56.5) vs 47.2% (33.2-59.3); p=0.19) (Figure 6.12).

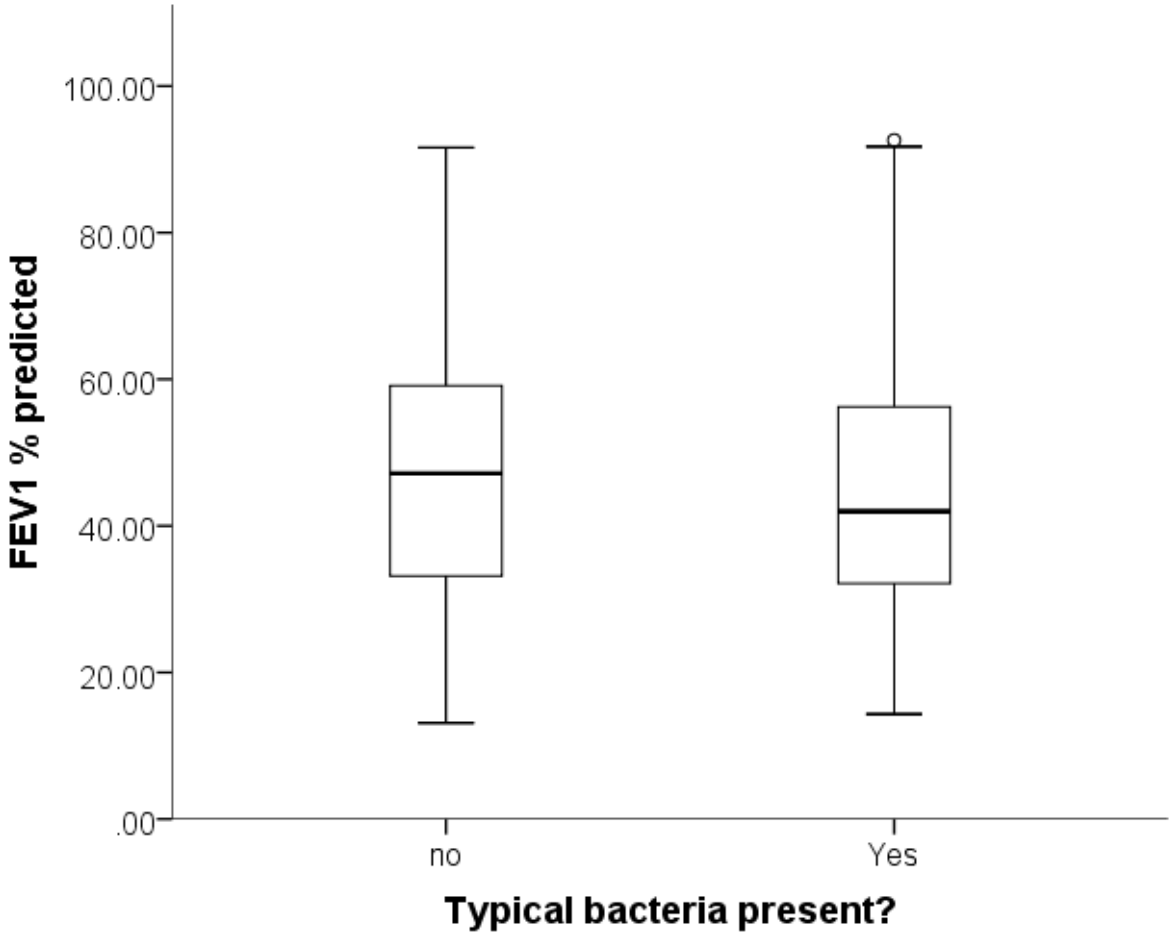


Figure 6.12. Airflow limitation measurements according to typical airway bacteria presence. No significant difference was identified.

However, in samples with typical airway bacterial presence, higher bacterial load did correlate with more severe airflow limitation during exacerbation and recovery ($r = -0.22$; $p=0.02$) (Figure 6.13).

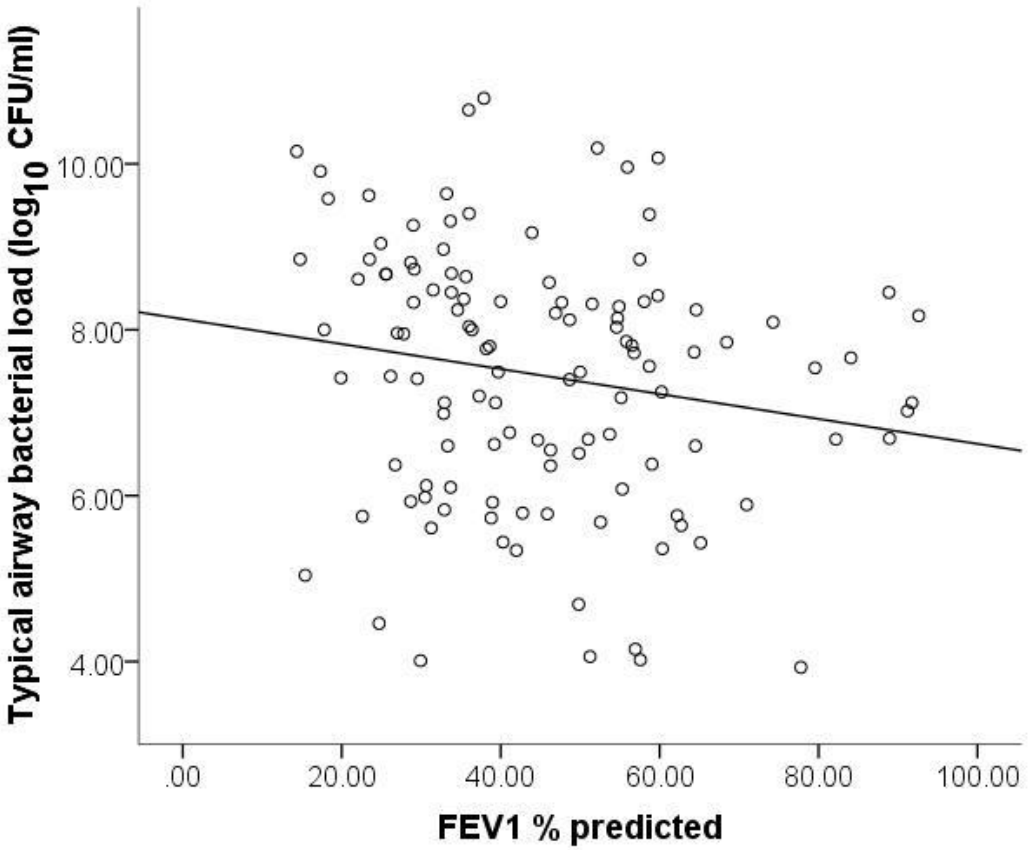


Figure 6.13. Relationship between typical airway bacterial load and airflow limitation, during exacerbation and recovery (n=118) (rho = -0.22; p = 0.02).

Having found that typical airway bacterial load related to airflow limitation, the load of the overall microbiome was then assessed with airflow limitation. No significant correlation was identified between microbiome load and airflow limitation (r=-0.12; p=0.11) (Figure 6.14).

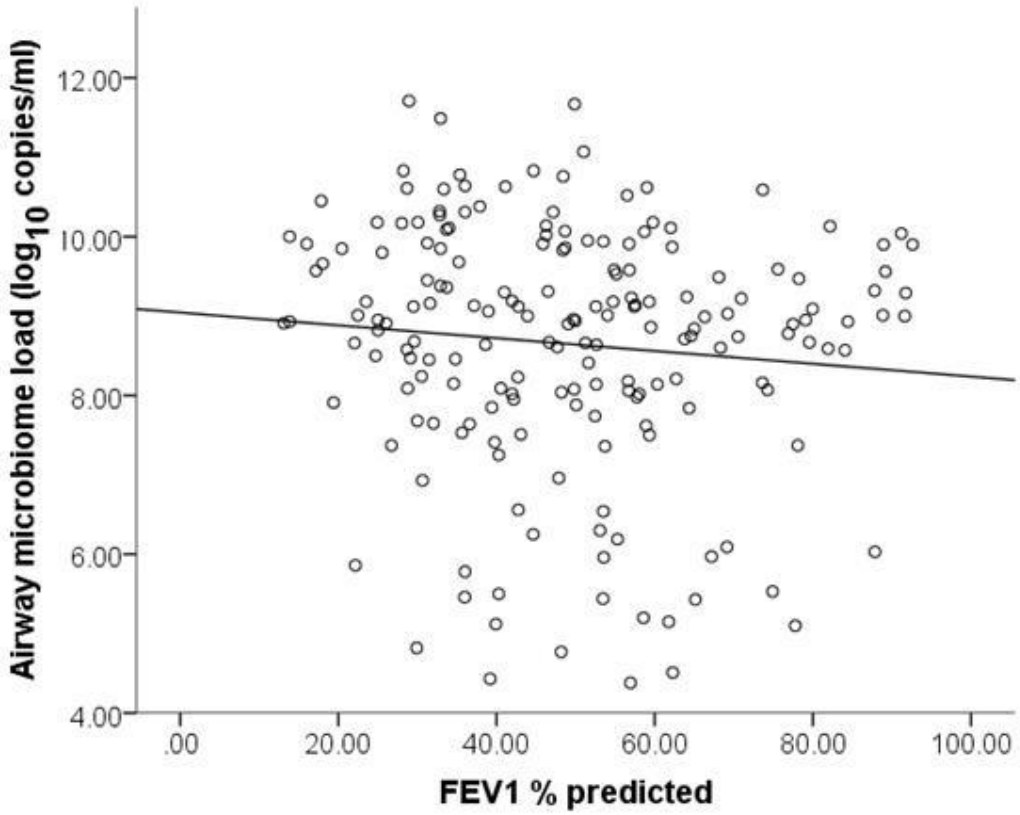


Figure 6.14. Relationship between airflow limitation and load of the airway bacterial microbiome (n=185). Microbiome load was not associated with airflow limitation (rho = -0.12; p=0.11).

6.3.8 Systemic corticosteroid prescription

Data on the prescription of systemic corticosteroids was available for 129 of the 135 exacerbation presentation visits (95.6%). In total, 105 exacerbations were treated with systemic corticosteroids (81.4%). Median systemic corticosteroid dosage was 30mg and median course length was 7 days. There was no difference in the median (IQR) serum CRP levels based on whether systemic corticosteroids were prescribed (13.5 (6-29) mg/L) or not prescribed (17 (6-49) mg/L) (p=0.69) (Figure 6.15).

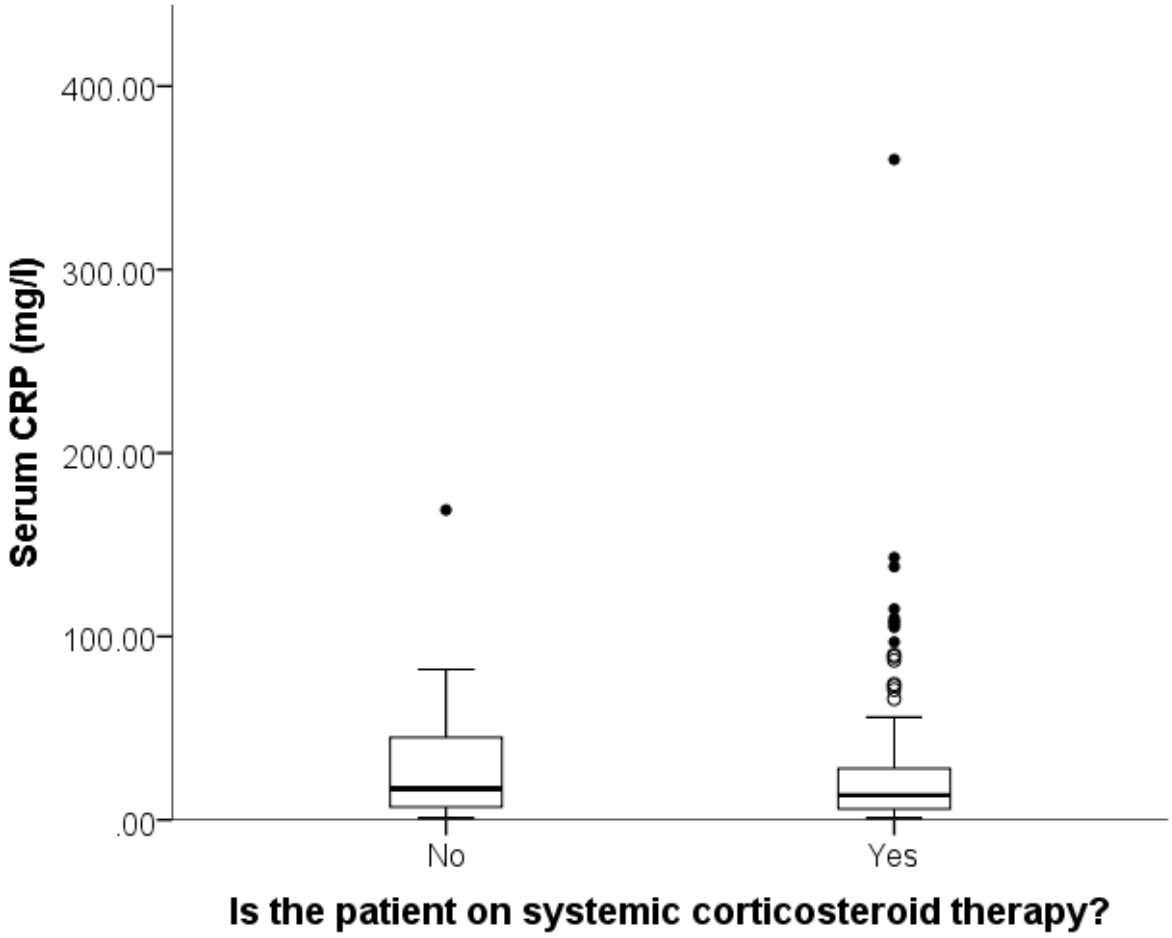


Figure 6.15. Serum CRP levels at exacerbation presentation sub-divided based on whether systemic corticosteroids were prescribed. No significant difference in CRP was identified between the two groups.

Additionally, there was no difference in prescription rates of systemic corticosteroid based on presence (81.2%) or absence (81.7%) of typical airway bacteria (p=0.94). Bacterial load at exacerbation presentation had no bearing on systemic corticosteroid dosing (r=0.025; p=0.77) (Figure 6.16).

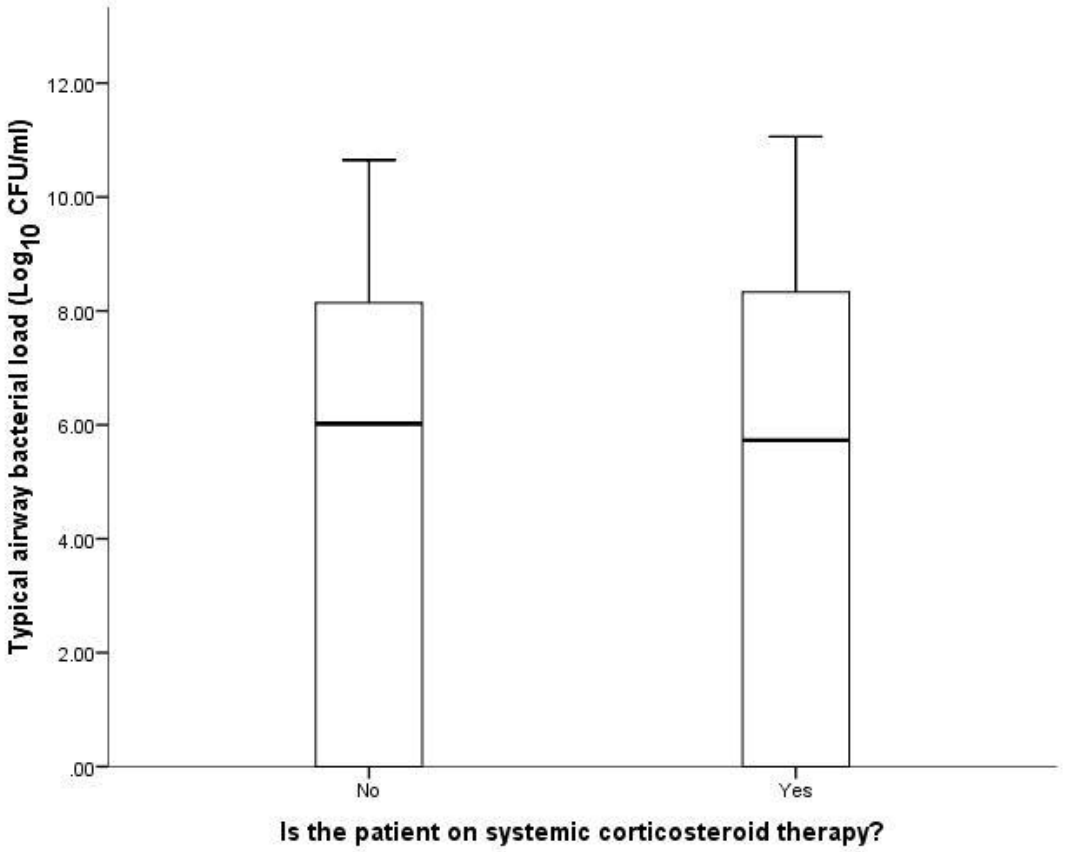


Figure 6.16. Relationship between typical airway bacterial load and systemic corticosteroid dose prescription at exacerbation presentation (n=129). No association between load and systemic corticosteroid prescription was seen (p=0.93).

There was no significant difference in median (IQR) load of the airway microbiome at exacerbation presentation, irrespective of whether systemic corticosteroids were prescribed ($10^{8.8\ (8.0\text{-}9.8)}$ copies/ml) or not ($10^{9.1\ (8.3\text{-}9.8)}$ copies/ml) (p=0.46).

There was found to be a significant correlation between systemic corticosteroid prescription and airflow limitation, with prescription associated with more severe limitation (p=0.03) (Figure 6.17).

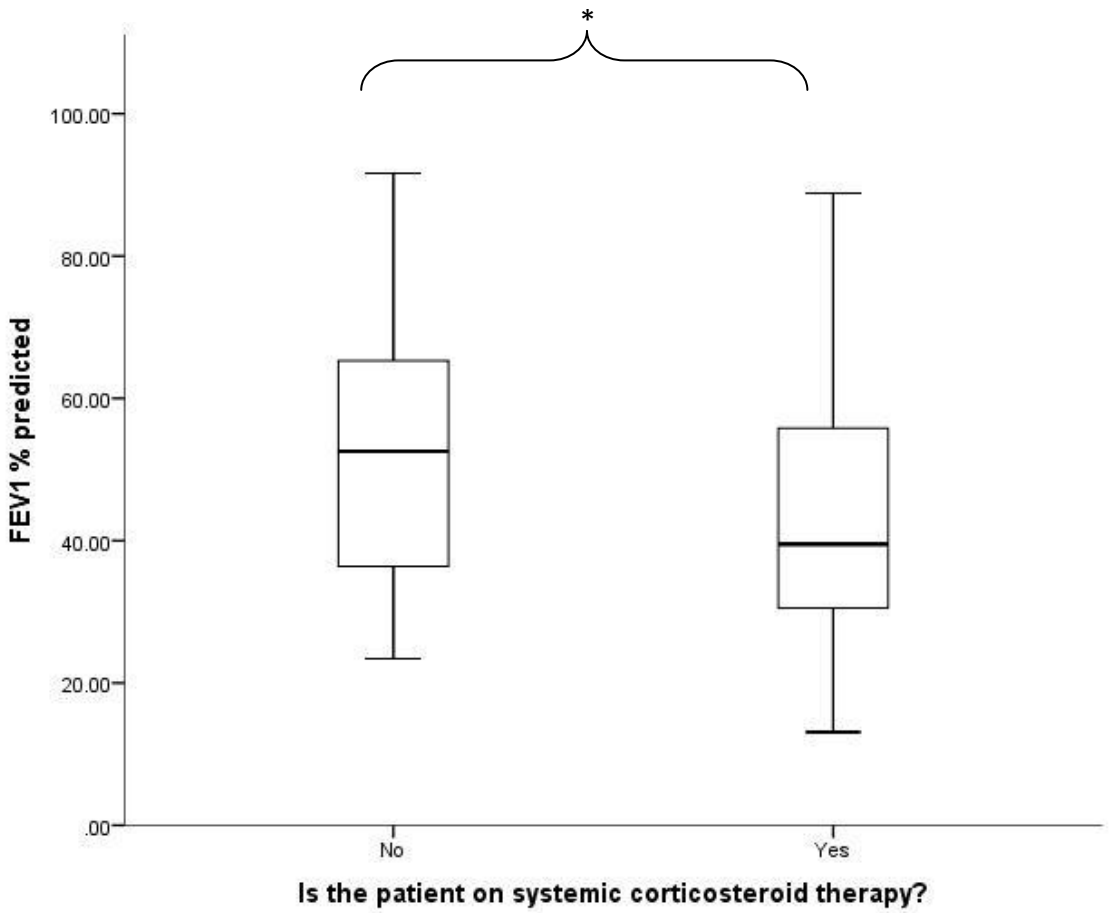


Figure 6.17. Relationship between airflow limitation and systemic corticosteroid dose prescription at exacerbation presentation (n=120). *p<0.05

6.4 Discussion

This investigation has shown, for the first time, that typical airway bacterial load falls significantly during recovery from an exacerbation over a 35 day period, following commencement of antibiotic therapy. There is also a significant fall in typical airway bacterial prevalence for at least 14 days post-exacerbation. Conversely, the airway microbiome is not eradicated following antibiotic therapy, demonstrating that the airways remain colonised with a commensal microbiota, although the load is reduced. It has also been demonstrated that systemic inflammation (as measured by the biomarker CRP) peaks at exacerbation before falling to stable state levels within 3 days of treatment. Systemic corticosteroid prescription and dosage was not associated with either systemic inflammation or with bacterial load at exacerbation presentation, although it was associated with airflow limitation. A plausible explanation for this may be that physicians use airflow limitation to guide dosage of systemic corticosteroids.

6.4.1 Typical airway bacterial load falls following antibiotic therapy

This study is the first to conclusively demonstrate, both in longitudinal and cross-sectional analyses, that typical airway bacterial load falls significantly following exacerbation presentation. The role of antibiotic treatment following exacerbation presentation may play a part in this fall, although it cannot be confirmed as the study was not placebo-controlled (which would have taken into account the effect of the immune system on load). The study has also shown that bacterial load is associated with systemic inflammation. Therefore from a clinical viewpoint,

antibiotic therapy, alongside systemic corticosteroids, may be an important means of controlling systemic inflammation in an exacerbating host.

6.4.2 Typical airway bacteria load is related to the load of the airway microbiome

The current study has, for the first time, compared the bacterial load of those bacteria frequently associated with COPD exacerbations (typical airway bacteria) with the load of the airway microbiome, which has in the last few years been found to be an inherent constituent of the human airways (Hilty et al. 2010; Huang et al. 2010; Erb-Downward et al. 2011; Sze et al. 2012). It has been hypothesised that this microbial community may contribute to disease pathogenesis and progression (Erb-Downward et al. 2012). Whilst the airway microbiome has been well defined as a component of both healthy and COPD airways, no studies up to now have explored changes in load. The current investigation is the first to examine the airway microbiome load in relation to typical airway bacterial load (Figure 6.7), demonstrating that in patients with typical airway bacterial presence, there is a positive correlation between airway microbiome load and typical airway bacterial load. This suggests that typical airway bacteria are not simply displacing the commensal microbiome population, but instead are growing in addition to it.

6.4.3 Typical airway bacteria dominate the load of the airway microbiome when present

Microbiome load was found to be six-fold higher in samples with typical airway bacteria than in those without. The implication of this data is that typical airway

bacteria flourish in addition to the commensal airway microbiome, rather than simply displacing it, and that this microbiota is dominated by typical airway bacteria.

6.4.4 Microbiome load falls following antibiotic therapy but it is not eradicated

Following presentation of an exacerbation, the microbiome load falls by eight-fold within three days post-therapy commencement, in a paired analysis. From the findings of this study, we also know that in paired samples, there is elimination of typical airway bacteria in the majority of patients within three days of treatment following a typical bacteria-associated exacerbation. It follows that elimination of typical airway bacteria contributes largely to the fall in microbiome load, particularly given that the microbiome load is six-fold higher when typical airway bacteria are present. However, the microbiome, which is known to consist of species as diverse as *Streptococcus, Prevotella, Pseudomonas, Moraxella, Haemophilus, Acinetobacter, Fusobacterium,* and *Neisseria* in COPD patients (Cabrera-Rubio et al. 2012; Huang et al. 2010), is never completely eliminated. This demonstrates that antibiotics do not target all species in this microbiota. The most frequently utilised antibiotic in this study was amoxicillin, either alone or in conjunction with the β-lactamase inhibitor, clavulanic acid. *Acinetobacter* and *Pseudomonas* are examples of genera of bacteria which are known to be resistant to the penicillin family of antibiotics (Shakibaie et al. 2012; Gad et al. 2008), and

they could be part of the core commensal species which remain despite antibiotic treatment.

6.4.5 Systemic inflammation biomarkers fall following commencement of systemic corticosteroids

Serum CRP was found to fall within three days of systemic corticosteroid therapy. It has previously been demonstrated that systemic corticosteroid usage significantly reduces median CRP levels in the seven days following an exacerbation presentation (Dentener et al. 2001). Given the previous findings in the current study that typical airway bacterial load also falls following exacerbation presentation, this decline in bacterial numbers may be another plausible mechanism to explain the decrease in systemic inflammation identified, and so it may not be solely an effect of the systemic corticosteroids.

6.4.6 Typical airway bacteria presence is associated with systemic inflammatory biomarker levels

Higher serum CRP levels were associated with presence of typical airway bacteria, providing evidence that serum CRP may be a useful clinical biomarker in the diagnosis of bacterial-associated exacerbations of COPD. This finding has been supported by work performed by Peng and colleagues in a Chinese population. They found that exacerbations with serum CRP levels in excess of 19.6 mg/L were associated with presence of bacteria, which they termed ‘potentially pathogenic

micro-organisms' (Peng et al. 2013). In the current study, we have also found that typical airway bacterial load is related to systemic inflammation. Typical airway bacteria have a number of virulence mechanisms which would cause this systemic inflammatory response, with presence of *Moraxella spp.* and *Haemophilus spp.* upregulating secretion of pro-inflammatory cytokines including IL-23 and IL-12 (Larsen et al. 2012). The pro-inflammatory response may be activated by detection of bacterial lipopolysaccharide, which stimulates an immune response (Moghaddam et al. 2011).

6.4.7 Airway microbiome load is not associated with systemic inflammatory biomarker levels

Given that there was a relationship between typical airway bacteria and systemic inflammation, the investigation then explored whether airway microbiome load was associated with a similar inflammatory response, using CRP. It was found that there was no such relationship. This illustrates that the typical airway bacteria cause a specific pathogenic response. It was also found that higher load of typical airway bacteria related to more severe airflow limitation, whilst there was no correlation between microbiome load and airflow limitation.

Taking these data together, there is evidence to suggest that during an exacerbation, typical airway bacterial species have pathogenic effects both locally in the airways and systemically via an increased inflammatory response, whilst the airway microbiome has little impact in terms of disease pathogenesis. The results

in this chapter do not consider the changes in the airway microbiome between stable and exacerbation state, and that is explored in Chapter 7.

At day 14 post-exacerbation presentation, median CRP levels increase to 8 mg/L, from just 3 mg/L at day 7. Given that it has previously been shown that CRP levels fall following systemic corticosteroid usage (Dentener et al. 2001), this rebound of CRP may be an effect of cessation of systemic corticosteroid therapy at day 7.

6.4.8 Typical airway bacterial prevalence falls following antibiotic therapy

Typical airway bacterial prevalence was found to almost halve in COPD patients, to 26.8%, in the three days following commencement of antibiotic therapy. This fall in prevalence was maintained for at least two weeks following an exacerbation. However at the five-week recovery time point, the prevalence reached 39.5%. It is possible that this increase in prevalence may be associated with recurrence of an exacerbation, as seen in 25% of the sample population. However, the frequency of recurrence is too low to confirm or refute this. Analysis of the population excluding recurrent exacerbations showed a decrease in prevalence of typical airway bacteria (39.5% to 35.3%). However this was still not significantly below prevalence of typical airway bacteria at exacerbation (prevalence of 51.5%).

Bacterial load was found to be significantly lower at all recovery time points compared to at exacerbation presentation, up to and including five weeks post-

presentation. Following on from the findings of Chapter 4, this demonstrates that there is a clear association between bacteria-associated COPD exacerbations and high bacterial load, relative to bacterial association at other time points.

This study showed that serum CRP peaked at exacerbation presentation before reducing significantly within 3 days. A previous study in the London COPD Cohort reported that in 77% of patients, symptoms recovered to baseline by day 35, and, as with this study, recovery was associated with a fall in CRP levels (Perera et al. 2007). The current investigation demonstrated that CRP fell markedly at days 3 and 7, before rebounding slightly at day 14 (still significantly below the level seen at exacerbation presentation).

The absence of a relationship between bacterial presence and recurrence of an exacerbation may be due to a number of reasons. It may be a further indicator of the efficacy of antibiotics at reducing bacterial load down to a level below the threshold required to trigger a bacterial exacerbation – this would be borne out by the evidence that bacterial load has been shown to fall in paired time course samples. It is also likely that many recurrent exacerbations may not be bacterial in origin. For example, whilst antibiotics may mitigate the threat from bacteria, opportunistic viral infections may be more likely to occur in a recovering host. However, this is speculative and further investigations would be required to ensure sufficient power to identify whether there is statistical significance.

6.4.9 Typical airway bacterial load is not associated with dosage levels of systemic corticosteroid

COPD exacerbations are routinely treated with systemic corticosteroids to control inflammation. As described earlier, systemic inflammation was associated with presence and load of typical airway bacteria. It was therefore hypothesised that typical airway bacterial load may be associated with dosage of systemic corticosteroids. However, no relationship between load and dosage was identified. This may be a consequence of the fact that the vast majority of exacerbations (81.4%) were treated with systemic corticosteroids, and there was little variability in dosing regimens. Dosage was, however, associated with airflow limitation at exacerbation presentation. This suggests that clinicians are using spirometry data to support dosing levels at time of an exacerbation.

6.5 Conclusion

This chapter has indicated for the first time that antibiotic therapy may be effective at limiting typical airway bacterial prevalence and load following an exacerbation whilst having a limited impact on the airway microbiome as a whole. Furthermore, it has shown that pathogenesis is associated specifically with these typical airway bacteria, and differences in the microbiome are likely of little significance in terms of pathogenic potential.

CHAPTER 7. **Changes in the airway microbiome between stable COPD and exacerbation**

7.1 Introduction

The airway microbiome can be described as the entire flora of airway micro-organisms. In this study, the airway microbiome relates specifically to bacterial micro-organisms. Until relatively recently, the lungs were considered to be maintained in a sterile state, or at most, 'insignificantly colonised' (Cabello et al. 1997). However, this orthodoxy has been overturned by molecular techniques such as plasmid-based sequencing, terminal-restriction fragment length polymorphism and microarray analysis (Hilty et al. 2010; Rogers et al. 2009; Huang et al. 2010), as well as by the evidence presented in Chapter 6 of the current study.

The human intestines are known to contain at least ten-fold greater bacterial cells than there are human cells in the entire body, constituting a total of 10^{14} bacterial cells (Festi et al. 2011). This gut microbiome is now known to be a vital component of digestion, allowing the degradation of plant polysaccharides and other dietary compounds, in a symbiotic interaction (Hooper et al. 2012).

The relationship of the host immune system with colonisation by the gut microbiome has been extensively studied in literature (Hooper et al. 2012; Festi et al. 2011). Understanding this relationship may provide a useful comparator to the interactions between the immune system and the airway microbiome. The majority of gastrointestinal tract microbiome species are strict anaerobes, with colonisation commencing at birth (Sekirov et al. 2010). The commensal intestinal bacterial species play an essential role in modulating the intestinal immune system,

ensuring survival of this microbiome. This 'cross-talk' between the host and these bacterial species is essential for a number of reasons. It allows maintenance of an intestinal barrier and provides an appropriate environment for appropriate immune system stimulation (Abt & Artis 2013). Maintenance of the intestinal barrier involves commensal species competing with pathogens, both for available nutrients and for attachment sites located on the intestinal brush border (Festi et al. 2011). It has, however, been acknowledged that this high density of micro-organisms may itself pose a threat to the host, via opportunistic invasion, potentially leading to inflammation and sepsis (Hooper et al. 2012).

In addition to the gut and airway microbiomes, the skin (Chiller et al. 2001; Percival et al. 2012) and genitourinary tract (Ma et al. 2012; Hyman et al. 2013) are also known to be host to bacterial microflora. A summary and brief description of known commensal microbiomes in humans is described (Table 7.1).

Microbiome location	Description	References
Gastrointestinal tract	Contains ~10^{14} bacterial cells. Majority of species are strict anaerobes and this microbiome plays an important role in digestion of plant polysaccharides. They also compete with pathogens, reducing the risk of infection	(Festi et al. 2011)
Skin	Commensal bacteria are largely gram-positive organisms, such as *Staphylococcus spp.* (primarily *S. epidermis*) and *Micrococcus spp.*. Commensal skin bacteria presence reduces the risk of cutaneous or systemic infection from pathogens	Chiller et al (2001); Percival et al (2012)
Genitourinary tract	The vagina is host to a commensal microbiome, largely characterised by the dominant presence of *Lactobacillus spp.* in the majority of women. *Lactobacilli* produce lactic acid, lowering the pH of the vagina - this provides a hostile environment for pathogens	Ma et al (2012); Hyman et al (2013)

Table 7.1 Commensal microbiomes of the human body

Due to the relatively recent identification of the airway microbiome, research is still at an early stage in terms of elucidating the role of this microbiome (Erb-Downward et al. 2012).

In 2010, Hilty and colleagues demonstrated that the bronchial tree contains a microbial flora which is characterised by the subject's health status (Hilty et al. 2010). It was later suggested that subjects with poor lung function exhibited

reduced microbial diversity compared to healthy smokers (Erb-Downward et al. 2011). However, this research contrasts with more recent findings by Pragman and colleagues, which indicated that COPD patients had a higher degree of microbiome diversity than controls (although in this case, not all controls were smokers) (Pragman et al. 2012). It has been acknowledged that there is limited evidence to support any functional role for the airway microbiome (Marsland et al. 2013).

Studies exploring the microbiome in COPD have focused particularly on diversity of species in this microflora, whilst the overall bacterial burden has not been investigated. The results presented in this chapter will explore the load of the airway microbiome at stable and exacerbation states to provide novel insights into the microbiome at these different stages of COPD.

7.2 Patient characteristics

We collected sputum from 47 patients in the London COPD cohort at stable state (n=32) and at exacerbation (n=68). Patient characteristics are illustrated in Table 7.2.

Characteristic	
Median age (range), years	70.7 (47.4-83.6)
Male gender (%)	63.8
Current smoker (%)	23.4
Median pack-year smoking history (IQR)	43.5 (16.4-60.5)
Mean FEV1 (SD), Litres	1.2 (0.5)
FEV1 % predicted (SD)	46.5 (18.7)
FEV1/FVC ratio (SD)	0.44 (0.1)
Mean FVC (SD), Litres	2.7 (0.9)

Table 7.2. Characteristics of 47 patients of the London COPD Cohort, who participated in a study of the airway microbiome at stable and exacerbation states. No significant differences are seen between these and the patients described in Table 3.5.

7.3 Results

7.3.1 Airway microbiome load in stable and exacerbated COPD

No significant difference was identified in microbiome load between the stable state (n=32) and exacerbated state (n=68) in an unpaired analysis ($10^{9.2(\pm0.2)}$ vs $10^{8.8(\pm0.1)}$ copies/ml; p=0.10) (Figure 7.1).

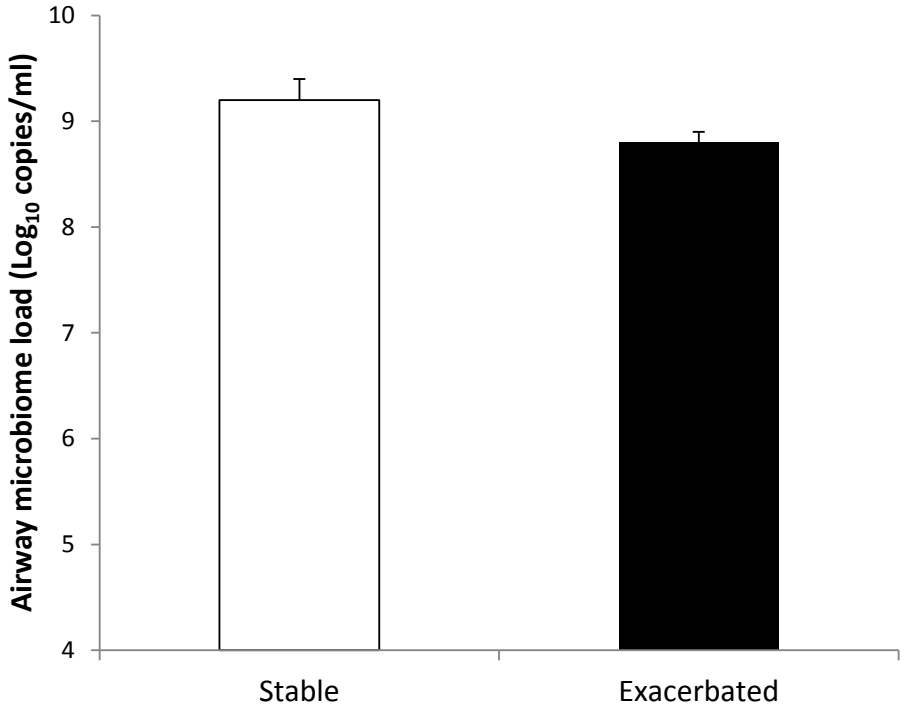


Figure 7.1. Airway microbiome load at stable (n=32) and exacerbation (n=68) states of COPD in an unpaired analysis.

In a longitudinal analysis involving 18 of these patients, airway microbiome load was assessed in both the stable and the exacerbation state, whereby the stable state sample was obtained less than 365 days prior to the exacerbation sample; median (IQR) = 119.5 (86.25-209.75) days prior.

As suggested with the inter-patient analysis above, it was found that there was also no significant intra-patient variation in airway microbiome load between stable and exacerbated state ($10^{9.2(\pm0.2)}$ vs $10^{8.8(\pm0.2)}$ copies/ml; p=0.13) (Figure 7.2).

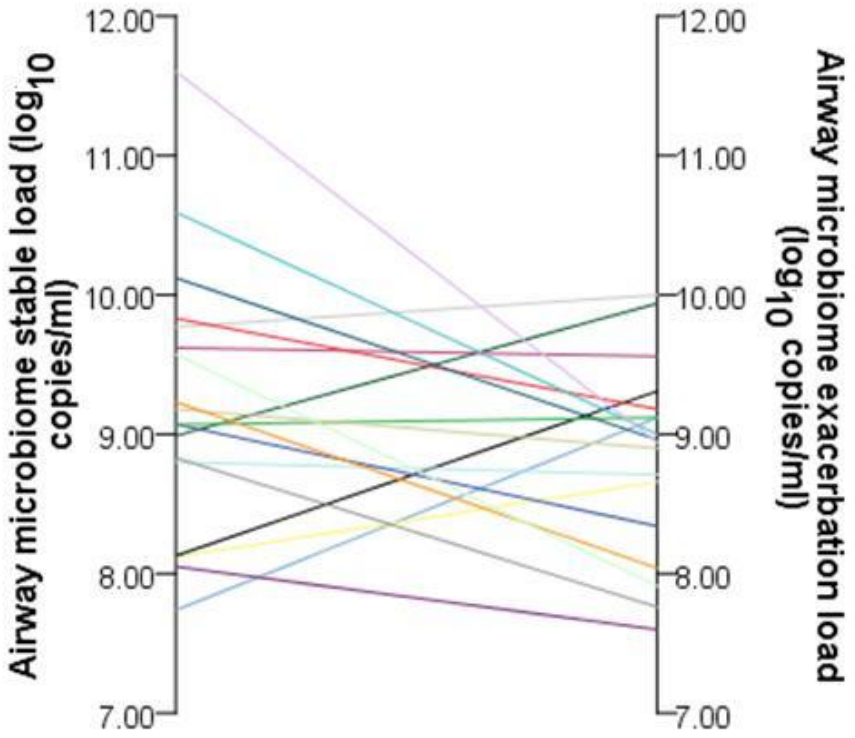


Figure 7.2. Intra-patient changes in airway microbiome load between stable and exacerbation states. Stable state sputum sample was obtained <365 days prior to exacerbation sample.

In stable COPD, there was found to be no significant difference in mean (SEM) load of the airway microbiome, irrespective of typical airway bacteria presence or absence ($10^{9.0(\pm0.3)}$ vs $10^{9.3(\pm0.2)}$ copies/ml, respectively; p=0.43) (Figure 7.3).

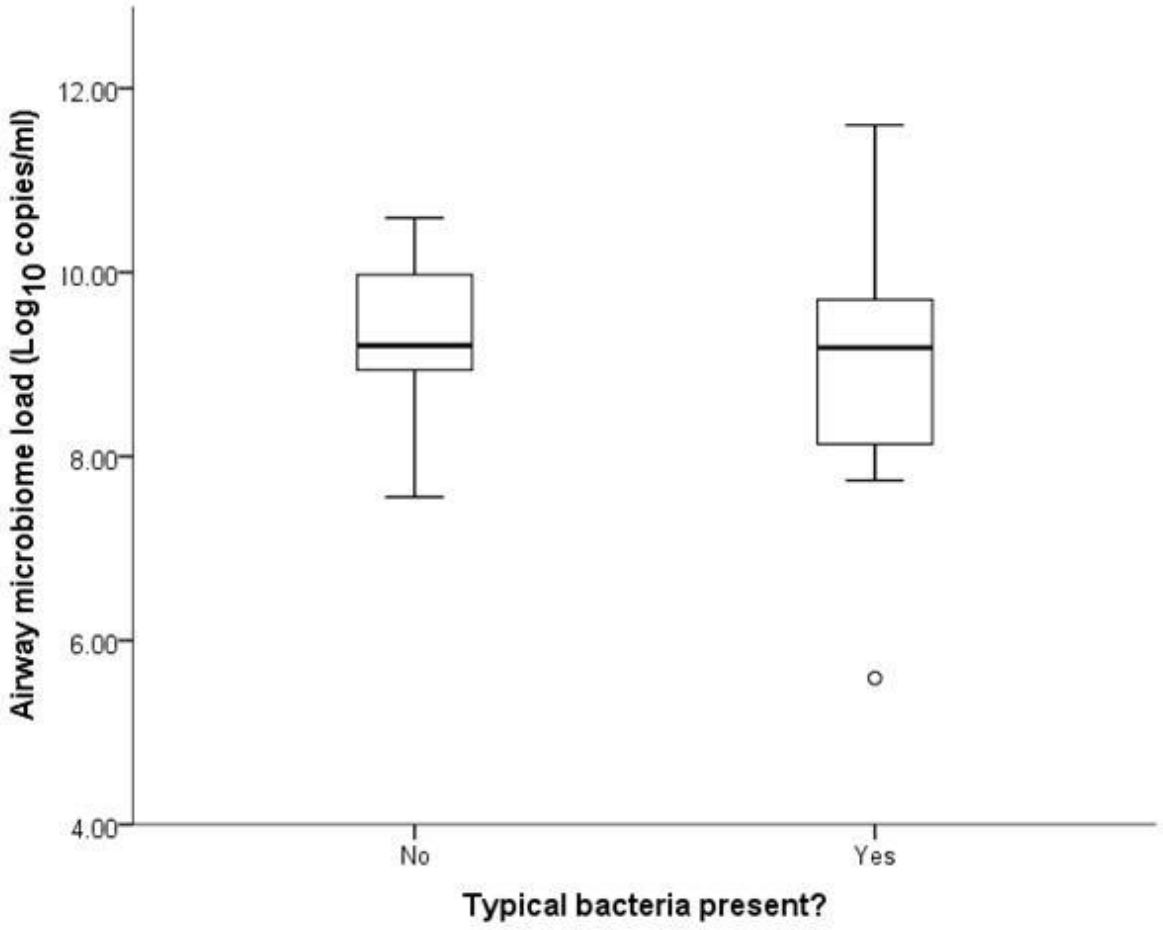


Figure 7.3. Airway microbiome load in the presence or absence of typical airway bacteria, in the stable state.

7.3.2 Airway microbiome load and airflow limitation at exacerbation

Airflow limitation data characterising the fall in FEV_1 at exacerbation compared to a previous stable state was assessed in 54/68 patients (79.4%). Patients were excluded from this subset if the exacerbation preceded any available stable spirometry data, if FEV_1 data was not available at both stable and exacerbation states, or if the stable state data was >365 days prior to the exacerbation sample.

The median (IQR) number of days separating the stable state reading from the exacerbation state reading was 99.5 (55.3-162.3) days.

The median (IQR) percentage change in FEV_1 at exacerbation was -5.6 (-20.6 to 3.4)%. There was found to be no significant correlation between microbiome load at exacerbation and change in FEV_1; rho= -0.04, p=0.76 (Figure 7.4).

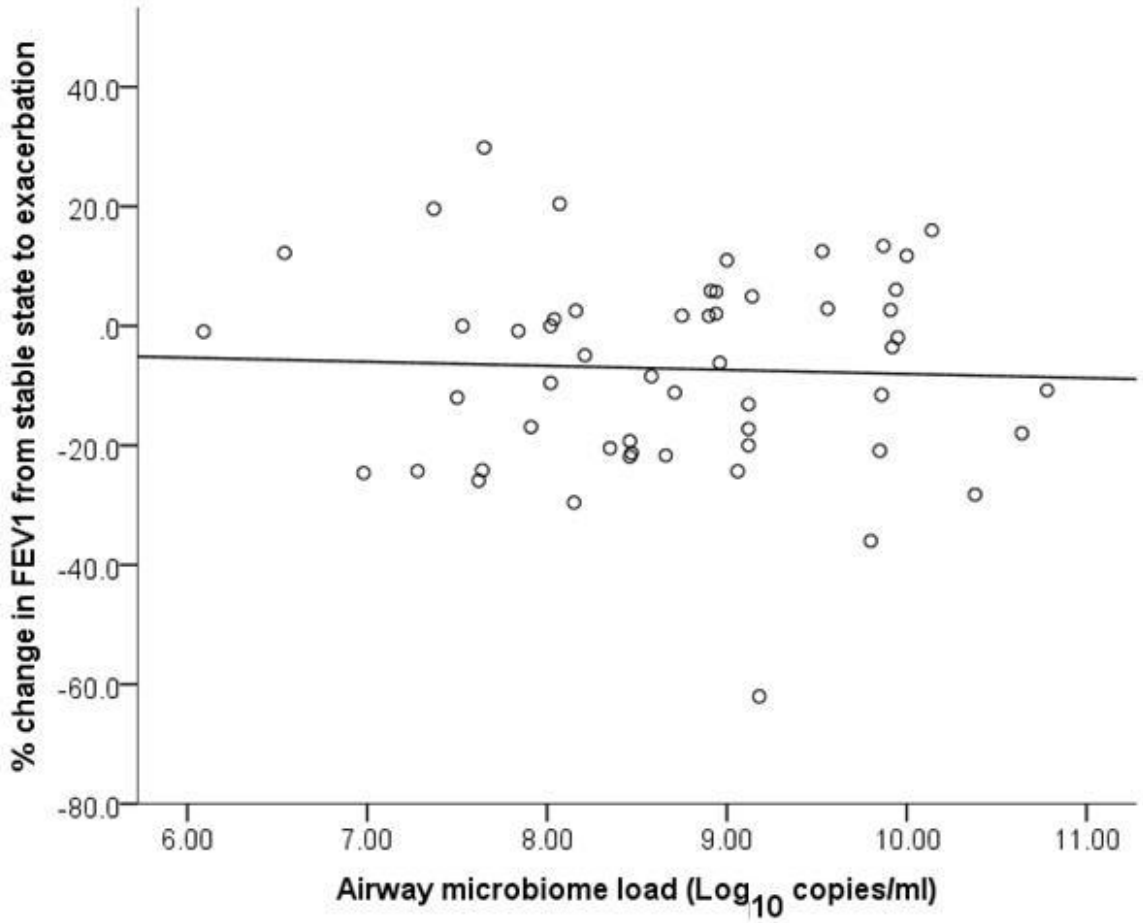


Figure 7.4. Airway microbiome load at exacerbation was not found to be related to FEV_1 fall from stable state to exacerbation, r= -0.04, p=0.76 (n=54)

This demonstrated that microbiome load at exacerbation was not related to changes in FEV_1 between stable and exacerbation states.

The previous microbiome analysis did not assess whether changes in the load of the microbiome between stable and exacerbation states related to changes in FEV_1. This was explored in those patients from whom stable and exacerbation state microbiome data had been obtained, and who also fulfilled the spirometry inclusion criteria as outlined earlier in this section. Fourteen of the 18 patients (77.8%) with paired data fulfilled these criteria.

The median (IQR) number of days separating stable state from exacerbation state was 119.5 (82.5-230.3) days. The median (IQR) percentage change in FEV_1 at exacerbation was -11.3 (-17.9 to 6.8) %. Twelve of these 14 patients (85.7%) demonstrated a fall in airway microbiome load at exacerbation compared to stable state. No significant correlation was identified between change in airway microbiome load and change in FEV_1 status, between stable state and exacerbation (rho= -0.15; p=0.62).

7.3.3 Airway microbiome load and sputum volume/purulence

Patient diary card availability allowed symptom data to be compared with airway microbiome load at exacerbation. Of the 68 exacerbations, 46 demonstrated an increase in sputum purulence. Sputum purulence at exacerbation was not associated with any difference in airway microbiome load, with mean airway

microbiome load of $10^{8.8(\pm 0.2)}$ cfu/ml, both with and without sputum purulence (Figure 7.5).

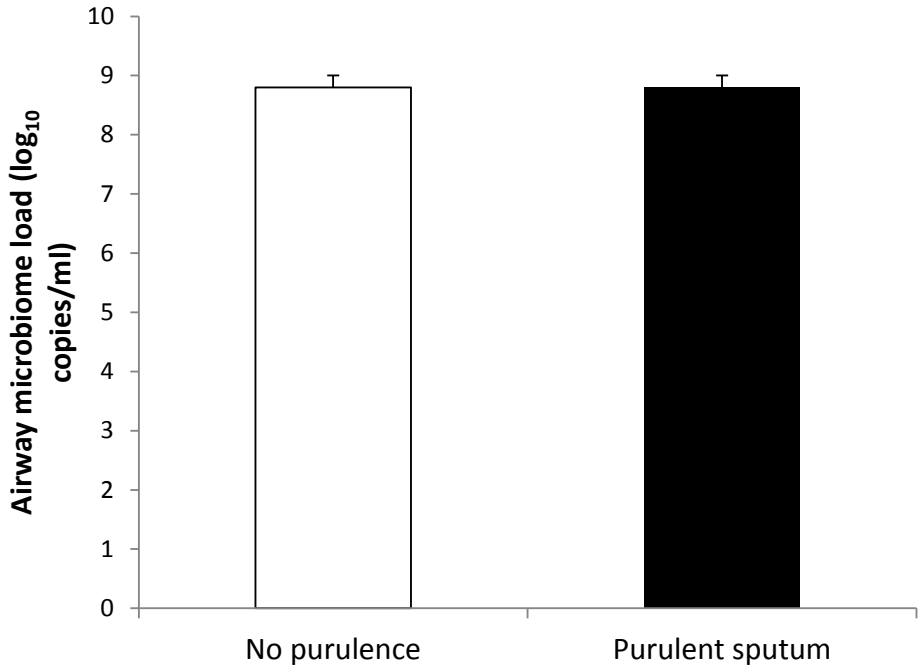


Figure 7.5. Airway microbiome load split by presence (n=46) or absence (n=22) of purulent sputum at exacerbation. No difference was seen in airway microbiome load, irrespective of sputum purulence

7.3.4 Relationship of airway microbiome load at stable and exacerbation state with biomarkers of systemic inflammation

Nineteen samples (19%) had levels of CRP recorded. No correlation was identified between airway microbiome load and CRP (rho= -0.05; p=0.85) (Figure 7.6).

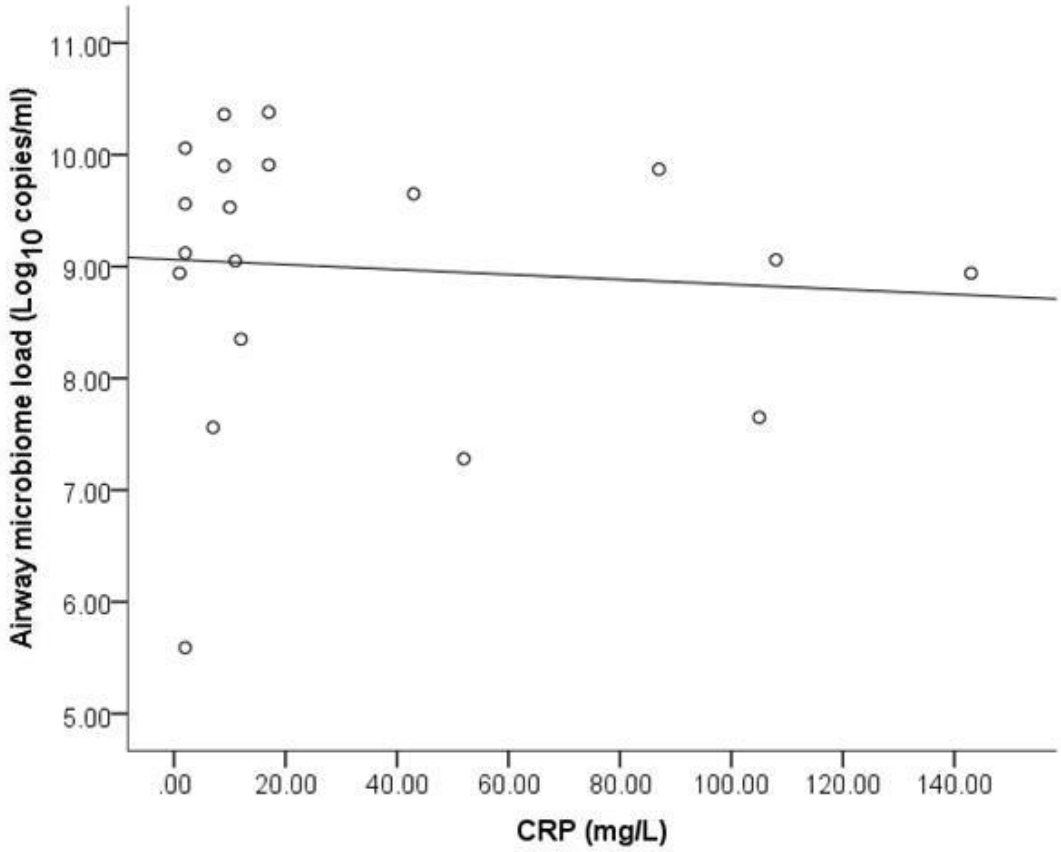


Figure 7.6. The relationship of airway microbiome load and CRP. (rho = -0.05; p=0.85)

7.4 Discussion

Bacterial microbiomes are known to be of importance in a number of different tracts and surfaces of the human body. The microflora of the gut, skin and genitourinary tracts has been well characterised (Festi et al. 2011; Chiller et al. 2001; Ma et al. 2012; Hyman et al. 2013). These characterisations have highlighted both the protective effects of these bacteria under normal conditions, as well as the pathogenic effects of opportunistic bacterial infection. However, the protective and/or pathogenic effects of the airway microbiome have not previously been investigated.

Current knowledge of the airway microbiome describes the diversity of bacterial species in considerable detail, looking at COPD patients, non-smokers and healthy smokers (Hilty et al. 2010; Huang et al. 2010; Erb-Downward et al. 2011; Sze et al. 2012). However, there had previously been no investigation into the changes of the airway microbiome load in COPD patients between the stable state and the exacerbation state. It was therefore important to do this investigation to establish if there was any evidence to indicate whether changes in the load of the airway microbiome have a protective or pathogenic effect in COPD.

In this study, a number of findings of the airway microbiome have been described for the first time. It has been shown that the microbiome is constitutively present in the airways of COPD patients and that the burden of this microbiome is not related to disease state. It was also found that there was no link between airway

microbiome load at exacerbation with changes in FEV_1 between exacerbation and the previous stable state. Furthermore, it has been demonstrated that microbiome load does not change significantly within patients from stable state to exacerbation state, showing that change in the microbiome load alone is not associated with disease severity. It is likely that the changes in the prevalence and load of particular species may be of more importance. It has been seen that in the stable state, presence of typical airway bacteria is not associated with a higher airway microbiome load. This contrasts with the findings at exacerbation, as described in section 6.3.4, where typical airway bacteria presence was associated with a six-fold higher airway microbiome load. No relationship was found between airway microbiome load and the systemic inflammatory biomarker CRP.

Sputum purulence is an indicator of bacterial infection in COPD, and is used as a guide for antibiotic therapy in COPD exacerbations (Stockley et al. 2000; Anthonisen et al. 1987). In order to assess whether or not the airway microbiome was a contributor to bacterial symptoms in COPD, the microbiome load was assessed in samples exhibiting or not exhibiting purulent sputum. There was found to be no difference in airway microbiome load, regardless of sputum purulence.

These data concerning the airway microbiome are in contrast to the previous findings highlighted in the current study examining the typical airway bacteria. Presence of the typical airway bacteria, are associated with a fall in FEV_1 from stable state to the following exacerbation, and the load of typical airway bacteria significantly increases at exacerbation (Chapter 4). Furthermore, typical airway

bacterial load was shown to be associated with CRP levels (section 5.3.2). It is also known that higher typical airway bacterial load is associated with sputum purulence (section 4.5.1).

Taken together this body of evidence indicates that typical airway bacteria prevalence and load, but not the airway microbiome, is associated with a more severe systemic inflammatory response and poorer airflow limitation, both at the stable state and at exacerbation. Therefore the importance of the airway microbiome may lie in changes in the presence and load of key bacterial species, of which the typical airway bacteria make up a major subset.

CHAPTER 8. **Prevalence and Load of Atypical Airway Bacteria at Stable and Exacerbation States of COPD**

8.1 Introduction

Atypical airway bacteria have previously been found to be causal agents of community-acquired pneumonia (Cunha 2006). However, they have previously been seldom identified in patients of COPD. In the field of COPD, atypical bacterial species which have been implicated in causing disease exacerbation are *Chlamydophila pneumoniae*, *Legionella pneumophila* and *Mycoplasma pneumoniae*.

Chlamydophila species exist in distinct forms depending on whether they are extra- or intra-cellular (Hammerschlag 2002). The free-living, extracellular form is known as an elementary body. In this state, the bacterium is able to infect mucosal epithelium cells. Once the bacterium is endocytosed it is contained within a phagosome, and this is prevented from fusing with the lysosome (this fusion would otherwise destroy the bacterium). These elementary bodies are then able to differentiate into reticulate bodies during conditions of immune stress. Reticulate bodies are forms of *Chlamydophila* species which exist and persist intracellularly. When there is no immune stress, the reticulate bodies can redifferentiate back into elementary bodies, triggering cell lysis and subsequent further infection (Kalayoglu et al. 2002).

In a similar mechanism, *Legionella pneumophila* (*L. pneumophila*) is able to colonise alveolar macrophages. This adaptation in *L. pneumophila* is thought to have been driven by its evolution in response to bacteria-consuming amoebae found in

aquatic environments, with significant conservation between macrophages and amoebae allowing *Legionella* to persist in both cell types (Molofsky & Swanson 2004).

Mycoplasma pneumoniae (*M. pneumoniae*) is one of the smallest known bacterium, with a genome size of 816 kb. Upon discovery, it was not readily identified as a bacterium and was termed Eaton's Agent, following discovery by Monroe Eaton and Colleagues in 1944 (Eaton et al. 1944). By the 1960's however, further analysis confirmed that it was indeed a bacterium, and it was ultimately given its current nomenclature (Chanock 1963). *M. pneumoniae* is able to infect and persist in cells of the respiratory epithelium (Collier & Clyde 1971; Waites & Talkington 2004).

8.1.1 Pathogenesis

Atypical airway bacteria have been shown to be a frequent cause of community-acquired pneumonia (Maartens et al. 1994). However, they act through differing mechanisms to produce this pneumonic phenotype.

Chlamydophila pneumoniae (*C. pneumoniae*; previously designated as *Chlamydia pneumoniae*) is a well-known cause of acute respiratory infection (Burillo & Bouza 2010). It is also a causative agent in bronchitis, pharyngitis and sinusitis (Blasi et al. 2009). Furthermore, recent studies indicate that *C. pneumoniae* is associated with both lung cancer and Alzheimer's disease (Zhan et al. 2011; Shima et al. 2010). In

COPD, it has been hypothesised that chronic *C. pneumoniae* infection may assist infection with different pathogens in the lower airways, enhance smoking-mediated inflammation in the bronchi and stimulate development of irreversible airway obstruction (Karnak et al. 2001).

Legionella pneumophila (*L.* pneumophila) is ubiquitous in freshwater environments, and can also survive in air-conditioning systems (Newton et al. 2010). Legionnaire's disease is a severe acute pneumonia caused by *L. pneumophila*, and is associated with smokers, the elderly and immunosuppressed individuals (Yu et al. 2004). There are a number of mechanisms by which *L. pneumophila* exerts its pathogenic effects: it causes pore formation in macrophages and erythrocytes, resulting in cytotoxicity (Kirby et al. 1998); it also has a number of virulence factors characteristic of most bacterial pathogens, such as lipopolysaccharide, flagella, pili and outer membrane proteins (Newton et al. 2010).

Mycoplasma pneumoniae targets respiratory epithelial cells for infection (Hu et al. 1977). The bacterium is able to produce hydrogen peroxide (as a final metabolite of glucose), leading to the lysis of erythrocytes (Arai et al. 1983). The release of hydrogen peroxide also triggers the production and secretion of interferon (IFN) by lymphocytes and phagocytes. IFN secretion is known to be involved in the pathogenesis of chronic inflammatory disease following its stimulation of macrophages. The reason for this is that activated macrophages secrete tumor necrosis factor (TNF), which triggers apoptosis (Yarilina & Ivashkiv 2010).

8.1.2 Atypical bacteria in COPD: Current knowledge

A number of techniques have been performed to attempt to identify presence of atypical airway bacteria in COPD. Routine microbiological culture is insufficient as standard culture media is not designed to encourage growth of these atypical airway bacteria. A number of studies have utilised serology to attempt to overcome this limitation (Lieberman et al. 2002a; Lieberman et al. 2001a; Lieberman et al. 2002b; Soler et al. 1998).

Serological analysis indicated a potential association of these atypical airway bacteria with acute exacerbations of COPD. These analyses were all based on positivity of sera to the suspected organism at different dilutions (titres). It was found that chronic *C. pneumoniae* infection was detected in 33.3% of COPD patients compared with 7% of controls (Lieberman et al. 2001a). In that study, chronic *C. pneumoniae* infection was defined as having specific IgG titre ≥1:28 and specific IgA titre ≥1:64. Furthermore, in 31 patients who had two hospitalisations in the study period, it was found that there was evidence of acute *C. pneumoniae* infection in 11.3% of hospitalisations (Lieberman et al. 2001a). Soler and colleagues identified presence of *C. pneumoniae* antibodies in 18% of patients suffering severe exacerbations of COPD, in whom a seroconversion titre ≥ 1:512 or an IgM titre ≥1:32 was used to define *C. pneumoniae* antibody positivity (Soler et al. 1998). Another serological study identified acute *C. pneumoniae* infection in 34% of COPD patients undergoing an acute exacerbation (Karnak et al. 2001), with the acute infection defined as having specific IgG antibody ≥ 1:512 or specific IgM antibody

≥1:16. In that study, non-COPD smokers were used as controls, and in that group only 5% were positive for *C. pneumoniae* antibody. These studies show that there is wide variation in detection of *C. pneumoniae* antibody in different populations.

Legionella spp. presence has also been investigated by serology, in a study which examined paired exacerbation and subsequent convalescence serum samples (mean 24.7 days apart) in COPD patients (Lieberman et al. 2002a). In 16.7% of paired samples, the antibody titre of either IgG or IgM was four-fold higher at exacerbation than at convalescence. In a control group consisting of post-trauma patients admitted to the hospital in the study period, only 3% showed a significantly higher titre of *Legionella spp.* at admission. *M. pneumoniae* (14.2%) antibodies (IgG, IgA or IgM) have also been detected in patients hospitalised with AE-COPD, with antibody titers defined according to commercially available kits used in that study (Lieberman et al. 2002b). The seroprevalence of *C. pneumoniae* and *M. pneumoniae* has also been established in stable COPD patients (Park et al. 2005). Seroprevalence to *C. pneumoniae*, defined as IgG titre ≥ 1:512 or IgM titre ≥ 1:20, was identified in 3.4% of patients. Seroprevalence to *M. pneumoniae*, defined as an antibody titre ≥1:64, was identified in 16.9% of patients, comparable to the seroprevalence identified in acute exacerbations of COPD (Lieberman et al. 2002b).

However, a more-recent PCR-based analysis performed in The Netherlands, examining 126 exacerbation sputum samples, showed no evidence of *C. pneumoniae* or *M. pneumoniae* infection, and only one case of *Legionella spp.* infection, with a non-pneumophila strain (Diederen et al. 2007). In that study,

Diederen and colleagues targeted a region of the outer membrane protein A gene of *C. pneumoniae*, a region of the macrophage infectivity potentiator (*mip*) gene for *L. pneumophila*, and a region of the P1 adhesin gene for *M. pneumoniae*, in three separate PCRs –TaqMan probes were utilised for detection and quantification.

8.2 Atypical respiratory PCR methodology

In the current study, a multiplex qPCR was utilised for the detection of atypical airway bacteria as described in section 2.8, and as previously detailed (Ling & McHugh 2013).

8.2.1 Analysis of patient samples for atypical respiratory bacteria at various time-points of disease severity

A total of 176 samples, from 80 patients of the London COPD cohort, were investigated for presence of the atypical respiratory bacteria (*Chlamydia pneumoniae, Legionella pneumophila, Mycoplasma pneumoniae*). Of these, 97 were stable samples and 79 were exacerbation samples. Patient characteristics were obtained through reference to the COPD cohort database (Table 8.1).

Characteristic	
Median age (range), years	71.8 (43.3-85.1)
Male gender (%)	66.7
Current smoker (%)	25
Median pack-year smoking history (IQR)	50 (35.25-72.5)
Mean FEV1 (SD), Litres	1.1 (0.5)
FEV1 % predicted (SD)	45.5 (17.8)
FEV1/FVC ratio (SD)	0.44 (0.1)
Mean FVC (SD), Litres	2.6 (0.9)

Table 8.1. Mean characteristics of patients whose samples were analysed for atypical bacteria (n=80). There were no significant differences between the characteristics of these patients and the characteristics of the patient characteristics described in Table 3.5.

8.3 Results

8.3.1 Atypical airway bacterial prevalence and load

In total, six atypical bacteria positive samples were detected, in six separate patients. Of these, five were *Legionella pneumophila* and one was *Mycoplasma pneumoniae* (Figure 8.1).

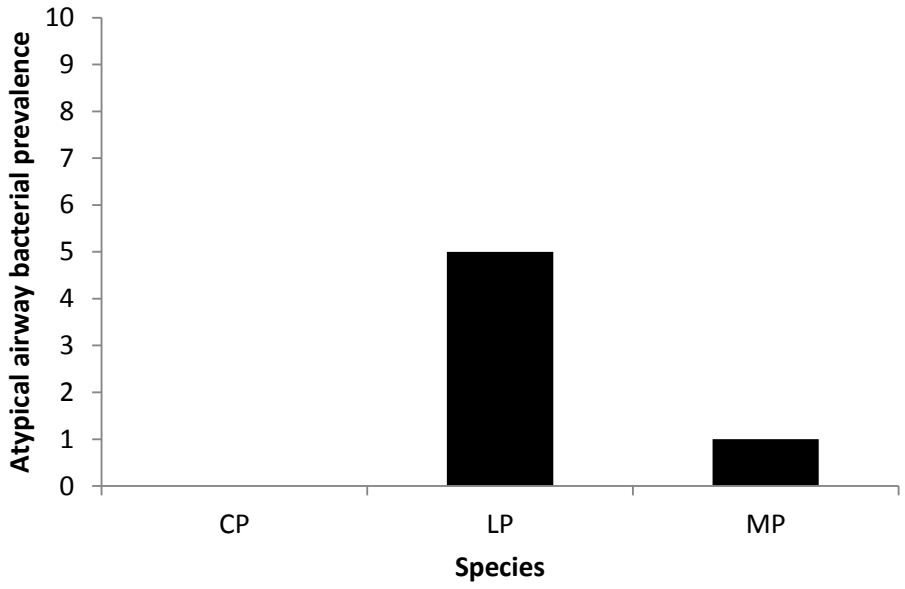


Figure 8.1. Prevalence of atypical airway bacteria in airways of COPD patients at stable (n=97) and exacerbation (n=79) states. CP = *Chlamydophila pneumoniae*, LP = *Legionella pneumophila*, MP = *Mycoplasma pneumoniae*

Bacterial load was quantified using the multiplex atypical airway qPCR (section 2.8), by correlating the positive samples to the standard curve of the respective species. *Legionella pneumophila* was found to have a mean (SEM) bacterial load of $10^{4.7\ (\pm 0.2)}$ CFU/ml (Figure 8.2). The five cases were found to range from $10^{4.3}$ CFU/ml to $10^{5.1}$ CFU/ml. Four of the positives were from stable samples and one was from an exacerbation sample. The highest bacterial load for *L. pneumophila* was from the

exacerbation sample. The single *Mycoplasma pneumoniae*-positive sample had a bacterial load of $10^{7.4}$ CFU/ml, and related to a stable sample.

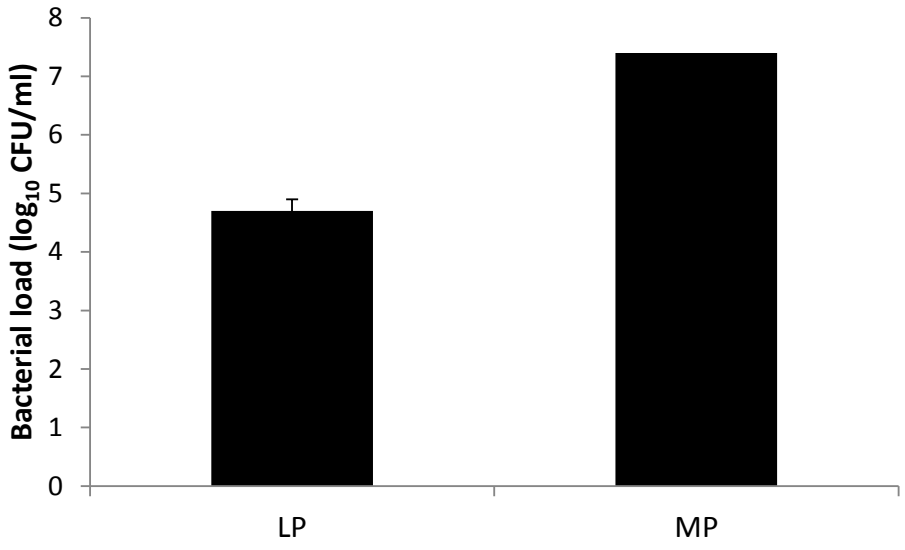


Figure 8.2. Bacterial load of patients with positivity for *L. pneumophila* (n=5) and *M. pneumoniae* (n=1). LP = *Legionella pneumophila*; *MP = Mycoplasma pneumoniae*.

8.4 Discussion

Detection of atypical respiratory bacterial infection is sought by methods other than culture. There are a number of disadvantages to using culture alone. For example, microbiological culture of *M. pneumoniae* is known to be laborious and expensive, with several passages required using specialised growth media and incubation periods of several weeks (Waites & Talkington 2004). Additionally, culture growth of *M. pneumoniae* has been shown to have only 61.5% specificity compared with PCR (Ieven et al. 1996). *L. pneumophila* growth is best identified using buffered charcoal yeast extract agar, and this growth can take at least three days to become apparent (Feeley et al. 1979). Alternative methods are therefore often employed for detection of such organisms. For example atypical airway bacteria are frequently detected by serology, as described in section 8.1.2. However, this technique is only able to detect presence of antibodies to these micro-organisms once seroconversion has occurred (i.e. antibodies to the respective bacterium develop). As such, antibody seroconversion can be an indicator of past infection as well as present infection, as antibodies remain present following resolution of an infection. Therefore it can be difficult to elucidate an association between serological detection and COPD exacerbations. Serology requires two sampling points 2-3 weeks apart, delaying diagnosis (McDonough et al. 2005).

In order to overcome these difficulties, molecular techniques such as PCR are considered more suitable to detect presence or absence of atypical airway bacteria at the time of sampling (Ieven et al. 1996; Cloud et al. 2000; McDonough et al.

2005). PCR is able to detect traces of DNA of these pathogens during an infection. Surfactant proteins in the lung assist clearance of exogenous DNA by macrophages, suggesting that any DNA detected relates to an ongoing infection (Palaniyar et al. 2005).

Serology-based studies have demonstrated that antibodies to atypical airway bacteria exist in a significant minority of COPD patients, in the region of 30% (Lieberman et al. 2001b; Murphy & Sethi 1992). However, Diederen and colleagues had previously explored the presence of atypical airway bacteria in stable and exacerbated COPD, finding little evidence of these organisms, in a population in the Netherlands (Diederen et al. 2007). In that study, there was detection of *Legionella spp.* (non-pneumophila) in 1/122 (0.8%) stable state sputum samples and detection of *Legionella spp.* (non-pneumophila) in 1/126 (0.8%) exacerbation state sputum samples. No detection of *C. pneumoniae* or *M. pneumoniae* was identified in any sample.

The evidence presented in this study supports the findings of the Netherlands study, with very low prevalence seen (6/176 samples, 3.4%). Furthermore, the current study is the first to describe the bacterial load in patients positive for atypical airway bacteria, showing that *L. pneumophila* was present at very low load. In comparison with the mean (SEM) load of typical airway bacteria identified in the stable state in section 4.3.4, the load of *L. pneumophila* was 300-fold lower ($10^{7.2(\pm0.1)}$ vs $10^{4.7\ (\pm0.2)}$ CFU/ml). Furthermore, the presence of atypical airway bacteria was not related to clinical symptoms and so suggests that this was sub-

clinical in most cases. One case of *L. pneumophila*-presence was associated with an exacerbation but no conclusions can be drawn from this as it was only a single case. These findings of low prevalence are further supported by a recent study performed by Perotin and colleagues in France, where they found no cases of atypical respiratory pathogens in 45 cases of acute exacerbations of COPD (Perotin et al. 2013).

The current study has investigated the rate of atypical airway bacteria in COPD patients. It has established that there is low prevalence and low load of atypical airway bacteria, and that presence of these bacteria was not related to clinical symptoms. It has therefore identified that atypical airway bacteria are not a major cause of pathogenesis in COPD, in either the stable or exacerbation state, and so do not require specific therapy.

CHAPTER 9. **Summary and Future Work**

The overall aim of this thesis was to investigate the relationship between bacterial presence and severity of COPD. Certain bacterial organisms (typical airway bacteria) have previously been shown to be associated with acute exacerbations of COPD. *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* are the pathogens most commonly associated with COPD exacerbations (Hunter & King 2001; Sethi et al. 2002). Molecular techniques such as PCR have come to the forefront of identification of bacterial species in a range of diagnostic scenarios (Chaidir et al. 2012; Curran et al. 2007; Brito et al. 2003). However, there was an absence of literature exploring changes in the prevalence and load of typical airway bacteria between the stable state and exacerbation state utilising PCR, as well as during the recovery period in the weeks following an exacerbation.

Previously there has been conflicting data of the association of the atypical airway bacteria, namely *Legionella pneumophila*, *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*, in COPD, and this required further analysis. The airway microbiome is now known to be an inherent component of both healthy and COPD lungs (Hilty et al. 2010; Erb-Downward et al. 2011; Sze et al. 2012). However, there has previously been no consideration of changes in the prevalence and load of this microbiome between stable COPD, acute exacerbation, and recovery.

Changes in prevalence and load of typical airway bacteria between stable and exacerbation states has previously been done before utilising culture, although this was performed either in different groups of patients at the two states (Monso et al. 1995; Rosell et al. 2005), or, in the case of a study involving paired stable and

exacerbation state samples from patients, it was only performed in exacerbations which resulted in hospitalisations, therefore representing more severe exacerbations, and involved stable state samples obtained post-exacerbation (Papi et al. 2006). The hypothesis was that prevalence and load of typical airway bacteria was higher at exacerbation, and that typical airway bacteria were associated with more severe clinical outcomes in COPD. The first strand of the investigation compared microbiological culture with PCR for the detection of typical airway bacteria. The second strand of the investigation utilised PCR for the detection and quantification of typical airway bacteria, atypical airway bacteria, and finally all bacteria of the airway microbiome. The findings of the study are summarised below.

9.1 Main Findings

- Conventional microbiological culture was compared with a quantitative multiplex PCR in *Chapter 3*. In a population of 439 sputum samples, it was found that qPCR identified more than double the number of typical airway bacteria compared with culture. PCR also has the benefit of being a more rapid technique, with results available on the same day as the sample is obtained, and may therefore be successfully utilised to identify typical airway bacteria in the sputum of COPD patients

- In *Chapter 4*, it was found that prevalence of typical bacteria was approximately twice as high during COPD exacerbation compared with at the stable state. This was identified both in an unpaired analysis (53.3% vs 33.6%; $p<0.001$), as well as in an analysis which utilised paired stable and exacerbation sputum samples from the same patient (68.9% vs 29.5%; $p<0.001$)

- *Haemophilus influenzae* was identified as the most prevalent typical airway bacterial species, both at stable state (20.0%) and at exacerbation (32.8%), as shown in *section 4.3.3*

- It was also found in *Chapter 4* that typical airway bacterial load was significantly higher at exacerbation than at stable state, with 10 patients in

whom typical airway bacteria was detected in both states showing a mean increase in typical airway bacterial load of 25-fold (p=0.02)

- In *section 4.4.4* it was seen that exacerbations with presence of *H. influenzae* were associated with a poorer outcome in lung function compared to non-*H. influenzae* associated exacerbations (-16.1% vs 1.7%; p=0.015)

- Median levels of the systemic inflammatory biomarker C-reactive protein were significantly higher at exacerbation compared to stable state (10.0 vs 3.0 mg/L; p<0.001), and this is illustrated in *Figure 4.8*

- At exacerbation, median levels of the systemic inflammatory biomarker C-reactive protein were significantly higher in samples exhibiting typical airway bacterial presence, compared to those in which no presence was detected (17.5 vs 3.0 mg/L; p=0.009), as shown in *section 4.5*

- In *section 4.5.1* sputum purulence was shown to be associated with typical airway bacterial load ($10^{8.0(\pm0.2)}$ CFU/ml with purulence, vs $10^{7.1(\pm0.3)}$ CFU/ml without purulence, p=0.01)

- It was found that inhaled corticosteroid dosage was significantly higher in patients with typical airway bacteria detected compared to those without typical airway bacteria in the stable state (2000 µg vs 1000 µg; p=0.018), and this is described in *section 5.4*

- In *section 5.4* it was also seen that there was a positive correlation between ICS dosage and typical airway bacterial load (rho = 0.20; p = 0.01)

- This correlation was particularly strong when only patients who were positive for typical airway bacteria were analysed (rho = 0.38; p= 0.01) (*section 5.4*)

- ICS dosage was shown to be related to GOLD stage (p=0.049), and ICS dose was also seen to be correlated to more severe airflow limitation (rho = -0.27; p=0.001), as measured by FEV_1, in *section 5.4.1*

- In *section 6.3.*2, it was seen that typical airway bacterial prevalence fell significantly during exacerbation recovery, from 50.4% at exacerbation presentation to 26.8% at day 3 post-therapy commencement (p=0.008), and this significant reduction in prevalence remained for at least 14 days (32.1%, p=0.02)

- The airway microbiome was found to persist despite antibiotic therapy following an exacerbation, although load does fall in the three days following therapy commencement ($10^{9.1(\pm0.1)}$ vs $10^{8.2(0.3)}$; p=0.02), and this is described in *section 6.3.3*

- Presence of typical airway bacteria in patients at exacerbation and recovery was associated with a six-fold higher median (IQR) airway microbiome load

compared to those patients without any presence of typical airway bacteria ($10^{9.7(\pm1.5)}$ vs $10^{8.9(\pm1.3)}$ copies/ml, $p<0.001$) and there was also a positive correlation between typical airway bacterial load and airway microbiome load, in patients positive for typical airway bacteria (rho=0.41; $p<0.001$), as noted in *section 6.3.4*

- In *section 6.3.5*, it was shown that load of *H. influenzae*, but not *S. pneumoniae* or *M. catarrhalis*, was found to be correlated to levels of CRP (rho = 0.21; $p<0.001$)

- Conversely in *section 6.3.5*, load of the airway microbiome was not found to have any correlation to CRP (rho = 0.021; p=0.77)

- In the five weeks following an exacerbation, CRP levels were significantly below those seen at exacerbation, as described in *Table 6.2*

- In *section 6.3.5* it was identified that airflow limitation was not related to CRP levels at exacerbation presentation (rho = -0.11; p=0.23)

- It can be seen in *Figure 6.13* that in those samples with typical airway bacteria, higher load of such bacteria during exacerbation and recovery is associated with more severe airflow limitation (rho = -0.22; p=0.02)

- Airway microbiome load showed no correlation to airflow limitation (rho = -0.12; p = 0.11), as described in *section 6.3.7*

- No significant difference in CRP levels were seen at exacerbation presentation between those patients who were (13.5 (6-29) mg/L) and those who were not prescribed (17 (6-49) mg/L) systemic corticosteroids (p=0.69), and this is described in *section 6.3.8*

- It can be seen in *section 7.3.1* that the load of the airway microbiome does not significantly differ between the stable state and COPD exacerbation ($10^{9.2(\pm0.2)}$ vs $10^{8.8(\pm0.1)}$ copies/ml; p=0.10), and this was confirmed using intra-patient data at the two states ($10^{9.2(\pm0.2)}$ vs $10^{8.8(\pm0.2)}$ copies/ml; p=0.13)

- *Figure 7.3* illustrates that airway microbiome load in stable COPD does not significantly change when typical airway bacteria is present or absent ($10^{9.0(\pm0.3)}$ vs $10^{9.3(\pm0.2)}$ copies/ml, respectively; p=0.43). This is in contrast to the findings of *section 6.3.4*, which had found that in exacerbation and recovery, typical airway bacterial presence is associated with a six-fold rise in airway microbiome load

- Airway microbiome load at exacerbation was not associated with change in FEV_1 between that exacerbation and a previous stable state FEV_1 reading (rho= -0.04, p=0.76), as described in *section 7.3.2*

- *Figure 7.5* illustrates that sputum purulence was not associated with airway microbiome load. Again, this is in contrast to the findings with typical

airway bacteria described in *section 4.5.1*, where it was shown that purulent sputum had a higher typical airway bacterial load than non-purulent sputum

- Atypical airway bacteria were detected, using qPCR, in 6/176 samples (3.4%) and this is described in *section 8.3.1*. *L. pneumophila* was detected in 5 samples, with *M. pneumoniae* detected in 1 sample

9.2 Additional findings

- There was no significant difference in airway microbiome load between those patients who suffered a recurrent exacerbation within 50 days of the index exacerbation, and those who did not, as shown in *section 6.3.6* ($10^{8.9(\pm0.3)}$ vs $10^{8.8(\pm0.2)}$ copies/ml; p=0.73)

- *Figure 6.11* illustrates that airflow limitation does not change significantly during the 5-week recovery period post-exacerbation

- In *section 6.3.7*, airflow limitation across the exacerbation recovery period was not found to be related to presence or absence of typical airway bacteria (42.0 vs 47.2% FEV_1% predicted; p=0.19)

- *Section 6.3.8* shows that typical airway bacteria presence or load has no effect on the systemic corticosteroid prescription or dose levels

- Airway microbiome load has no bearing on whether or not systemic corticosteroids are prescribed, as detailed in *section 6.3.8*

- Higher systemic corticosteroid dosage was found to be related to more severe airflow limitation at time of exacerbation presentation (rho = -0.25; p=0.01) as illustrated in *Figure 6.17*

- Airway microbiome load in the stable state was not related to CRP (rho= -0.05; p=0.85), as shown in *section 7.3.4*

- Mean load of *L. pneumophila* was found to be $10^{4.7\ (\pm 0.2)}$ CFU/ml, as shown in *figure 8.2*

9.3 Clinical implications of findings

- The initial finding of this study verified the utility of quantitative PCR as a more sensitive method than microbiological culture for the detection of typical airway bacteria (*Chapter 3*). Conventionally, microbiological culture has been used as the primary method for the detection of airway bacteria. There are a number of disadvantages associated with the use of culture. Culture takes days, or even weeks to identify slow-growing organisms, and this may require highly selective media (Prakash et al. 2013). In clinical practice, this may delay necessary antibiotic treatment. Also, competition between bacterial species for the nutrients provided may result in one species outcompeting other species, thereby preventing their growth (Hibbing et al. 2010). There are also advantages to utilising culture, and this study by no means attempts to devalue the technique. For example, microbiological culture can be used to quantify antibiotic resistance in colonies and is also able to identify species that may otherwise not be looked for.

  For the purposes of this study, the aim was to identify the prevalence and load of known typical and atypical airway bacteria, or the entire airway microbiome. Quantitative PCR was therefore the most appropriate technique and is also likely to be of benefit in routine clinical practice when specific bacterial species are being targeted.

- There was previously doubt about the association of typical airway bacteria with exacerbations of COPD. This doubt was primarily due to the fact that typical airway bacteria are also isolated from sputum of stable COPD patients (Hirschmann 2000). The current study provides evidence to implicate typical airway bacteria in contributing to disease severity in COPD. Firstly, it has been shown that the prevalence of typical airway bacteria is significantly higher at exacerbation than at stable state. Secondly it has been shown that load of typical airway bacteria was 25-fold higher in patients suffering an acute exacerbation compared to the load found in those same patients at stable state less than one year prior. These two components indicate that typical airway bacteria are associated with exacerbation. Further evidence shown in this study went on to demonstrate how the typical airway bacteria, particularly *H. influenzae*, may be contributing to disease severity: at exacerbation, typical airway bacterial presence was associated with significantly higher levels of the systemic inflammatory biomarker, CRP, compared with samples in which no typical airway bacteria were detected; *Haemophilus influenzae*-associated exacerbations were associated with a much greater fall in airflow limitation from stable state compared to other exacerbation types.

  Whilst the evidence implicates typical airway bacterial prevalence and load in severity of COPD, there was no evidence to suggest that changes in the airway microbiome as a whole contributes to severity. Airway microbiome load was not associated with levels of CRP, or with sputum purulence, suggesting that neutrophilic infiltration is not increased when microbiome load increases.

There was also no association between airway microbiome load and airflow limitation. Therefore, the findings of this study suggest that antibiotic therapy targeting the typical airway bacteria is of particular importance in reducing the severity of COPD.

- It was found that exacerbation recovery was associated with a significant reduction in both the prevalence and load of typical airway bacteria, in the days following presentation with an exacerbation of COPD. This is associated with antibiotic treatment, and placebo-controlled studies would demonstrate whether the antibiotics are responsible for this reduction. A reduction in CRP was also identified over this time period, although that was presumably due to the anti-inflammatory nature of systemic corticosteroids also prescribed – the reduction in typical airway bacterial load may also play a part in this. It is therefore important to consider the potential benefit of prophylactic antibiotic therapy as a pro-active approach to prevent typical airway bacteria from infecting COPD patients.

Previous studies have demonstrated that long term antimicrobial therapy is of benefit to patients to COPD. Examining the macrolide class of antibiotics, two major studies examined long term antibiotic therapy, finding that daily administration of erythromycin and azithromycin for 1 year reduced exacerbation frequency compared to placebo (Seemungal et al. 2008; Albert et al. 2011). Given that macrolides have both antibiotic and anti-inflammatory capabilities, it was not clear by which mechanism they were acting.

Intermittent fluoroquinolone therapy has also been investigated in the PULSE study (Trial No. NCT00473460). In this trial, patients received moxifloxacin once-daily for 5 days (Sethi et al. 2010). This was repeated every 8 weeks for 48 weeks. It was shown to reduce odds of exacerbation by 20% in the intention-to-treat population. Encouragingly, there was also no evidence of development of resistance. A recent study explored the efficacy of antibiotics in mild-to-moderate exacerbations of COPD, finding that therapy with co-amoxiclav had a higher recovery rate than placebo (74.1% vs 59.9%) and had a longer median time to next exacerbation (233 days vs 160 days). The current study suggests that by reducing infection with typical airway bacteria, inflammation may also be directly reduced, providing clinical benefit.

- With typical airway bacteria being shown to be associated with acute exacerbations of COPD in this study, the attention must in future turn towards exploring vaccine development for COPD patients. *Streptococcus pneumoniae* vaccines are currently available and in the UK this is recommended for COPD patients, as was discussed in *Chapter 3*. The difficulty with *S. pneumoniae* is in the number of different serotypes, with more than 90 known (Weinberger et al. 2011). The pneumococcal vaccine, however, only targets 23 of these serotypes. A recent trial has indicated that oral therapy with inactivated non-typeable *H. influenzae* may show efficacy at reducing mild-to-moderate COPD exacerbations by 63%, in 38 patients (Tandon et al. 2010). The authors acknowledged that they needed to explore efficacy in a larger group representing a broader range of subjects with COPD.

9.4 Conclusion

This study has explored the role of airway bacteria in chronic obstructive pulmonary disease. It has demonstrated that typical airway bacteria are more prevalent and seen at higher load at exacerbation than stable state, and also present a greater burden of symptoms than atypical airway bacteria or the airway microbiome. Furthermore, antibiotic therapy at exacerbation presentation is associated with a marked decline in typical airway bacteria prevalence and load. Therapy aimed at alleviating bacteria-associated symptoms in COPD patients should be primarily targeted towards the typical airway bacteria.

9.5 Future Work

- It has not yet been conclusively demonstrated whether typical airway bacteria are causing exacerbation or whether they are simply involved in contributing to severity once an exacerbation arises. Analysis of samples taken at the 'stable' state in the prodrome phase prior to an exacerbation will provide evidence as to whether or not typical airway bacteria increase in this phase, and may therefore be triggering an exacerbation.

- In an experimental model it has been shown that viral infection induces an acute exacerbation and this leads to an increase in the load of the airway microbiome (Molyneaux et al. 2013). It would be of interest to identify, in a natural model, whether viral infection leads to an increase in typical airway bacterial prevalence, as antibiotic therapy may then prove to be a useful means to treat virus-associated exacerbations.

- Long-term antibiotic therapy trials have shown a reduction in exacerbation frequency following treatment. An examination of exacerbation frequency in relation to typical airway bacterial load would indicate whether it is an antibiotic or an anti-inflammatory mechanism which is resulting in this reduction.

- Sequencing of bacterial species which are detected in sputum would be useful as it would allow strain identification to occur and this could show whether or not strain switching is contributing to the increase in load seen at exacerbation.

- Given that typical airway bacterial load was higher at exacerbation than at stable state, further analysis may help determine the load at which typical airway bacteria trigger clinical symptoms such as CRP increase and airflow limitation.

- Sequencing of the airway microbiome may be a useful means to identify if any species is having a protective effect i.e. disease severity is higher in its absence. This may pave the way for probiotic therapy.

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APPENDIX

NAME	
Study Number	

THE LONDON COPD STUDY

Nov 2013	NEXT APPOINTMENT
WORSENING SYMPTOMS? CALL US	/ /13 . am

DATE	1 fri	2 sat	3 sun	4 mon	5 tue	6 wed	7 thu	8 fri	9 sat	10 sun	11 mon
Peak Flow											
CHANGE in Symptoms											
CHANGE in Treatment											
Hours out of the home											

DATE	12 tue	13 wed	14 thu	15 fri	16 sat	17 sun	18 mon	19 tue	20 wed	21 thu	22 fri
Peak Flow											
CHANGE in Symptoms											
CHANGE in Treatment											
Hours out of the home											

DATE	23 sat	24 sun	25 mon	26 tue	27 wed	28 thu	29 fri	30 sat
Peak Flow								
CHANGE in Symptoms								
CHANGE in Treatment								
Hours out of the home								

Instructions for filling in the DIARY CARDS

EVERY DAY...

1. After taking morning medications record the best of 3 attempts at the PEAK FLOW blowing test in the box on the sheet.
2. Please record any **WORSENING** of symptoms **ABOVE YOUR USUAL** daily level. The symptoms we are interested in are listed below, just put the appropriate letter in the box on the sheet. Continue recording until the symptom has gone away or got back to the level you consider 'normal'.

Letter	Symptom
A	increased BREATHLESSNESS.
B1	increased SPUTUM COLOUR.
B2	increased SPUTUM AMOUNT.
C	a COLD (such as a runny or blocked nose).
D	increased WHEEZE or CHEST TIGHTNESS.
E1	SORE THROAT.
E2	increased COUGH.
F	FEVER.

If you experience a worsening in any of these symptoms please phone us to arrange an assessment visit, and do this BEFORE starting any antibiotic or steroid tablets. The phone number is

07762 038662.

Anant or Alex will have the phone and we can usually arrange to see you later the same day or the following morning.

Please phone if you are not sure what to write down or you have any questions.

3. Please record any **CHANGE** to your usual treatment for as many days as it applies. Again, just put the appropriate letter in the box on the sheet.

Letter	Treatment
H	I am in Hospital.
I	I am taking more than usual INHALED STEROID (red / brown/purple)
R	I needed to take extra RELIEVER (blue / green / grey / nebuliser). **HOW MANY PUFFS?** Write, eg 'R3' for 3 puffs, 'R2' for 2 etc
S	I am taking STEROID (Prednisolone) TABLETS. **HOW MANY TABLETS?** Write, eg 'S6' for 6 tablets, 'S5' for 5 etc
X	I am taking ANTIBIOTIC TABLETS. **PLEASE RECORD WHICH** (write the name on the diary card).

4. Finally, please estimate the time that you were out of your own home on the previous day.

ORIGINAL ARTICLE

Changes in prevalence and load of airway bacteria using quantitative PCR in stable and exacerbated COPD

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► An additional appendix is published online only. To view this file please visit the journal online (http://dx.doi.org/10.1136/thoraxjnl-2012-201924).

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ABSTRACT

Background Prevalence and load of airway bacteria in stable and exacerbated chronic obstructive pulmonary disease (COPD) has been previously studied using microbiological culture. Molecular techniques, such as quantitative PCR (qPCR), may be more informative.

Methods In this study, 373 sputum samples from 134 COPD outpatients were assessed for prevalence and load of typical airway bacteria (Haemophilus influenzae, Streptococcus pneumoniae, Moraxella catarrhalis) by multiplex qPCR, with 176 samples analysed for atypical bacteria. Paired stable and exacerbation typical bacteria data were compared in 52 patients. We compared routine culture with qPCR in 177/373 samples.

Results Typical bacteria were more prevalent in exacerbation than stable-state paired samples: 30/52 (57.7%) vs. 14/52 (26.9%); p=0.001. In patients who were bacteria-positive at both time points, mean (±1 SEM) load was significantly higher at exacerbation than stable state ($10^{8.5(\pm0.3)}$ vs. $10^{7.2(\pm0.5)}$ cfu/ml), constituting a 20-fold increase (p=0.011). qPCR was more discriminatory at detecting typical bacteria than microbiological culture (prevalence 59.3% vs. 24.3%; p<0.001). At stable state, higher airway bacterial load correlated with more severe airflow limitation (FEV_1% predicted) (r=−0.299; p=0.033) and higher inhaled corticosteroid dosage (r=0.382; p=0.008). Mean C-reactive protein was higher in bacterial-associated exacerbations (35.0 Vs 25.1 mg/L; p=0.032).

Conclusions Airway bacterial prevalence and load increase at COPD exacerbations and are an aetiological factor. qPCR is more discriminatory than culture, identifying higher airway bacterial prevalence. Exacerbations associated with bacterial detection showed a higher mean C-reactive protein level. In the stable state, airway bacterial load is related to more severe airflow limitation and higher inhaled corticosteroid dosage used.

Key messages

What is the key question?

- Is there a change in prevalence and load of airway bacteria in COPD patients between the stable state and exacerbation, and what is the association between bacteria and lung function, inhaled corticosteroid dosage and systemic inflammation?

What is the bottom line?

- Airway bacterial prevalence and load, as detected by qPCR, increases significantly at COPD exacerbation, with quantitative molecular techniques offering a more accurate reflection of prevalence and load compared with conventional methods. Higher bacterial load is correlated with poorer lung function and higher inhaled corticosteroid dosage in stable COPD patients; bacterial presence at exacerbation is associated with higher systemic inflammation.

Why read on?

- Increased airway bacterial load is a key aetiological factor in acute exacerbations of COPD, and also relates to disease severity in the stable state. Inhaled corticosteroid usage may affect bacterial colonisation, and thus increase susceptibility to pneumonia.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is an inflammatory condition caused by an abnormal response to noxious gases and particles.[1] COPD is associated with considerable morbidity and mortality and has been projected to become the fourth highest cause of death by 2030.[2] Acute exacerbations of COPD are episodes which are characterised by a worsening of respiratory symptoms associated with variable degrees of physiological deterioration.[3] These events are a major cause of morbidity and mortality globally, and COPD exacerbations are a leading cause of hospital admissions.[3]

Lower airway bacterial colonisation (LABC) is a common and important feature of COPD, and the commonly isolated organisms are *S pneumoniae*, *H influenzae* and *M catarrhalis*,[4 5] although most studies investigating LABC have used culture techniques. LABC has been shown to be related to an increase in airway inflammation, exacerbation frequency and lung function decline.[5–8]

Previous studies suggest that the majority of exacerbations have an infectious aetiology—with approximately half these events associated with culture of potentially pathogenic airway bacteria, similar to those found in the stable state.[9] Positive cultures of *Pseudomonas aeruginosa* have also been shown to be associated with COPD, but more commonly in advanced disease.[10]

To date, the vast majority of analysis of bacterial prevalence and load has been based on culture, but there is little on PCR comparison with culture. The use of culture-independent techniques, such as quantitative PCR (qPCR), has not been extensively studied in the context of atypical airway bacteria, which are characteristically difficult to culture (*C pneumoniae*, *L pneumophila* and *M pneumoniae*), with one previous study showing no association with COPD exacerbations.[11] Conversely, serological analysis has suggested an association,[12 13] and further examination is warranted.

The objective of this study was to investigate the prevalence and load of typical and atypical airway bacteria in COPD using modern molecular techniques. We also analysed prevalence and load at the stable and exacerbation states, and compared qPCR with routine microbiological culture.

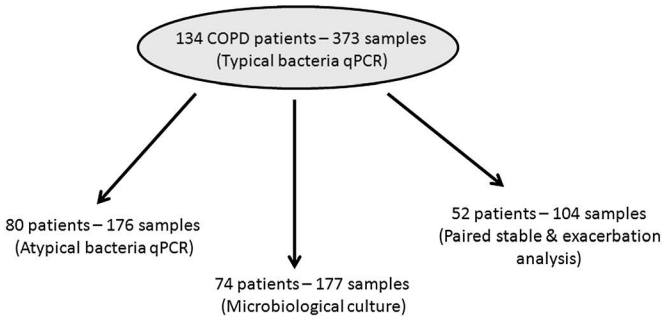

Figure 1 COPD patient groups analysed. Patients may have been in more than one subanalysis. For paired analysis, stable samples were obtained <365 days prior to exacerbation (the nearest stable sample to the exacerbation was used).

METHODS

Recruitment criteria

This study involved 134 subjects enrolled in the London COPD cohort between January 2007 and March 2011. The patients form part of a rolling cohort used to prospectively investigate the mechanisms and aetiology of COPD exacerbations, as previously described by our group.[14] Patients were included if the forced expiratory volume in one second (FEV_1) was ≤80% predicted and FEV_1/forced vital capacity (FVC) ratio was <0.7, in keeping with GOLD stages II–IV.[1] A history of chronic symptoms (dyspnoea, sputum production, wheeze and cough) was taken, as well as smoking history (number of pack-years smoked, current smoking status). Patients with a history of any other significant respiratory diseases were excluded, as were those unable to complete daily diary cards.

Definitions of clinical states

An exacerbation was defined as new or increased respiratory symptoms for two or more consecutive days, with at least one major symptom (dyspnoea, sputum purulence and sputum volume), and another major or a minor symptom (wheeze, cold, sore throat and cough), the first day of which was defined as the day of onset of the exacerbation. Stable state was defined as no symptom-defined exacerbations for the preceding 4 weeks and the subsequent 2 weeks post-clinic visit.

Clinical assessment

Spirometry was performed in accordance with ATS/ERS guidelines, using a volumetric storage spirometer (Vitalograph 2160, Maids. Moreton, Buckingham, UK). FEV_1 was expressed as a percentage of that predicted from age, height and sex. Serum C-reactive protein (CRP) quantification was performed to assess systemic inflammation, using Modular Analytics E 170 Module (Roche, Burgess Hill, UK). Patients recorded inhaled corticosteroid dosage and usage daily on diary cards. Due to variations in dosage by different medications, we applied beclomethasone-equivalence dosage correction, as previously performed by our group.[15]

Sputum collection and processing

Sputum was spontaneously expectorated into a sterile pot in the stable state and/or at exacerbation. All exacerbation visits occurred within 7 days of exacerbation symptom onset. Patient samples were used in a number of different analyses (figure 1).

Following expectoration, sputum plugs were separated from saliva using sterile forceps, with one-third being taken for culture analysis where there was sufficient quantity. The remaining sputum was weighed and suspended in nine times volume of standard isotonic phosphate-buffered saline (PBS). Glass beads were added to this suspension and it underwent homogenisation by vortexing for 15 s, rocking for 15 min (IKA Vibrax VXR, Staufen, Germany) and additional vortexing for 15 s. Aliquots of 500 µl were taken for storage at −80°C for subsequent batch processing.

Routine microbiological culture

Qualitative bacterial culture was carried out by the Department of Medical Microbiology, Royal Free Hampstead NHS Trust, London. Briefly, sputum was cultured onto suitable agar for 24 h at 37°C and 5% CO_2. Agar types used were Columbia blood agar (general growth media), Chocolate agar (growth of fastidious bacteria), MacConkey agar (growth of Gram-negative bacteria), and COBA agar (growth of *Streptococcus* spp.). *S pneumoniae* was identified through optochin sensitivity testing.

DNA extraction

Homogenised sputum samples underwent heat-kill treatment at 90°C for 30 min before being microfuged at 13 000 g for 10 min. The pellet was washed in 1 ml PBS and spun at 13 000 g for 10 min before removal of supernatant and re-suspension of the pellet in 200 µl of PCR-grade UV-sterilised water (Sigma-W4502). To each sample, 200 µl of 10% Chelex 100 (Sigma C-7901) was added. Samples were incubated for 20 min in a heat block at 56°C. Samples were heated at 95°C for five minutes prior to cooling on ice. Samples were then spun in a microfuge at 16 000 g for 10 min. Supernatant, containing extracted DNA, was transferred to a fresh UV-sterilised 1.5 ml tube and stored at 4°C.

Multiplex qPCR detection of bacteria

Real-time multiplex qPCR was performed on *S pneumoniae*, *H influenzae* and *M catarrhalis*, targeting Spn9802 (gene fragment), *H influenzae* P4 lipoprotein gene, and *copB* outer-membrane-protein gene, respectively. Both Spn9802[16] and *copB*[17] profiles used previously validated qPCR primers and probes, ensuring no cross-reactivity with related oral species. The *H influenzae* P4 lipoprotein gene target was established in-house. This was confirmed to have no cross-reactivity with other *Haemophilus* species as well as a number of *Streptococcus* spp., *M catarrhalis, Bordatella pertussis, Candida* spp., *P aeruginosa, Escherichia coli, Neisseria meningitidis, Acinetobacter* spp., *Legionella pneumoniae, Klebsiella pneumoniae*, methicillin-resistant *Staphylococcus aureus* and *Corynebacterium diphtheriae*. An internal amplification control (IAC), targeting the

Table 1 Stable-state clinical characteristics of COPD patient subgroups

	COPD patient subgroups				
	Typical bacteria analysis (n=134)	Culture analysis (n=74)	Atypical bacteria analysis (n=80)	Paired analysis (n=52)	p Value
FEV_1 (l)	1.22 (±0.52)	1.20 (±0.5)	1.11 (±0.50)	1.15 (±0.50)	0.407
FEV_1/FVC (%)	45.8 (±12.9)	45.2 (±12.6)	43.8 (±13.4)	43.4 (±13.4)	0.596
Predicted FEV_1 (%)	48.5 (±18.1)	48.5 (18.3)	45.4 (±17.8)	46.4 (±17.5)	0.582
Current smoker (%)	28	32	25	29	0.860
Age (years)	69.8 (±8.7)	69.8 (±8.3)	70.1 (±8.9)	68.7 (±8.5)	0.840
Male sex (%)	64	69	68	61	0.773
Pack years smoking	52.5 (±34.6)	55.9 (±38.2)	57.1 (±34.9)	42.8 (±24.2)	0.118

No significant differences were seen between the groups. Paired analysis refers to patients with paired stable and exacerbation-state samples (the nearest stable-state sample prior to exacerbation was used in this analysis). Data is presented as percentage value or mean±SD as appropriate.

PhyB gene of *Solanum tuberosum*, was used in order to detect any PCR inhibition, incorporated in the PCR mastermix.[18] The following positive control strains were used: *S pneumoniae* ATCC strain 49619; *H influenzae* ATCC strain 10211; *M catarrhalis* ATCC strain 25238. The minimum limit of detection used in this study was 10^4 colony-forming units (cfu)/ml.

A separate multiplex qPCR was performed to detect *C pneumoniae*, *L pneumophila* and *M pneumoniae*, targeting RNA-polymerase β-chain gene, MIP gene and P1 adhesin protein gene, respectively. *C pneumoniae* primer/probe set was a previously validated set.[19] *M pneumoniae* and *L pneumophila* were developed in-house and shown to have no cross-reactivity with related species and other oral flora. The PCR was performed with two types of mastermix, one of which was spiked with an IAC (*L pneumophila* DNA). Primer and probe details are listed in web-only files in the appendix. Normalisation of qPCR results was performed by correcting Ct values of the IAC in positive samples according to its corresponding value in no-template-control samples.

Statistical analysis

Data were analysed using PASW Statistics V.18 (SPSS Inc.). The Kolmogorov–Smirnov test for normality was applied. Clinical data with normal distribution are described in mean (±SD). Differences between groups were analysed by independent-samples t test, paired-samples t test, Mann–Whitney U test or one-way ANOVA, dependent upon the sample population being investigated. Relationships between continuous variables were analysed using Pearson's or Spearman's correlation in a univariate analysis, or by linear regression in a multivariate analysis. Frequency distribution was explored by χ^2 analysis. A probability of $p<0.05$ was considered to be statistically significant.

Ethical considerations

The study was approved by the Royal Free Research Ethics Committee, and all patients gave informed written consent.

RESULTS

Patient characteristics

Sputum samples from 134 COPD patients were examined. There were no clinically or statistically important differences in stable-state clinical characteristics between the subgroups (table 1).

Typical bacteria prevalence and load determined by qPCR

Typical bacterial prevalence by qPCR was higher in exacerbation sputum samples compared with stable samples (74/131 bacterial isolates (56.5%) vs 107/242 (44.2%) respectively, p=0.024).

In samples positive for bacteria, mean (±1 SEM)-typical bacteria count was significantly higher during exacerbation, at $10^{8.3(\pm0.2)}$ cfu/ml (in absolute numbers 2×10^8 cfu/ml), compared with the stable state, with $10^{7.3(\pm0.2)}$ cfu/ml (in absolute numbers 2×10^7 cfu/ml), a 10-fold increase, and significantly different ($p<0.001$) in an unpaired analysis (figure 2). *H influenzae* was the most prevalent species, both in the stable state (21.9%) and at exacerbation (26.0%). *M catarrhalis* was significantly more prevalent at exacerbation (16.8%) compared with the stable state (7.4%) (p=0.005). Mean (±1 SEM) bacterial load of both *S pneumoniae* and *H influenzae* was significantly higher at exacerbation than at stable state: $10^{8.1(\pm0.3)}$ vs $10^{7.2(\pm0.3)}$ cfu/ml, p=0.043 and $10^{8.2(\pm0.2)}$ vs $10^{7.0(\pm0.2)}$ cfu/ml, $p<0.001$, respectively.

Typical bacteria prevalence and load in paired stable and exacerbation samples

Fifty-two pairs of stable and exacerbation samples from 52 patients showed significantly higher cumulative prevalence of typical bacteria at exacerbation compared with the stable state (67.3% vs 30.8%; $p<0.001$). Fourteen stable-state samples (26.9%) were positive for at least one bacterium compared with 30 exacerbation-state samples (57.7%) (p=0.001). In paired samples which were bacteria-positive at both stable and exacerbation state (n=12), the mean load was approximately 20-fold higher at exacerbation than in the stable sample ($10^{8.5(\pm0.3)}$ vs $10^{7.2(\pm0.5)}$ cfu/ml) (p=0.011) (figure 3B).

The species detected with highest prevalence was *H influenzae* at stable state (11/52, 21.2%), with *S pneumoniae* most frequently

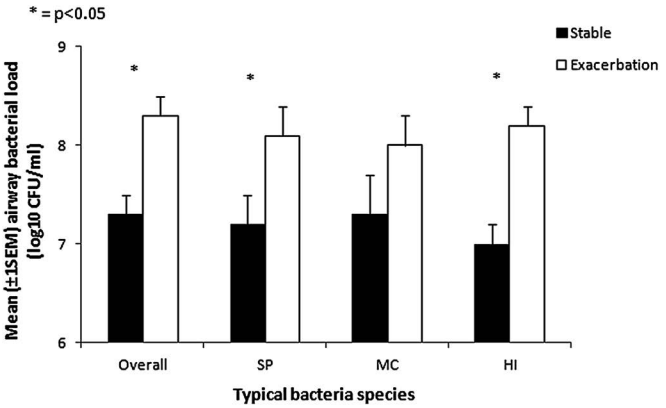

Figure 2 Quantitative PCR comparison of load of typical airway bacteria in stable COPD (n=242) and exacerbation (n=131) in unpaired samples. Species: SP = *Streptococcus pneumoniae*; MC = *Moraxella catarrhalis*, HI = *Haemophilus influenzae*.

seen at exacerbation (13/52, 25%) (figure 3A). *M catarrhalis* showed a significant increase in prevalence at exacerbation (10/52, 19.2%), increasing from 2/52 (3.8%) at the stable state (p=0.014). *S pneumoniae* prevalence was significantly higher at exacerbation (13/52, 25.0%) than during stable state (3/52, 5.8%) (p=0.002).

Comparison of qPCR with culture for the detection of typical bacteria

Culture and qPCR for typical bacteria were compared in 177 samples (105 stable and 72 exacerbation samples) from 74 patients.

qPCR was far more discriminatory at detecting typical bacteria than culture (105/177 positive samples (59.3%), vs 43/177 (24.3%), p<0.001). This finding was replicated in terms of individual bacterial species: *H influenzae* 30.5% PCR-positive versus 15.8% culture-positive, (p=0.001); *S pneumoniae* 14.1% versus 5.1%, (p=0.004); *M catarrhalis* 14.7% versus 3.4%, (p<0.001) (figure 4).

Positive culture identification and negative qPCR identification was seen for *H influenzae* in 4/177 samples (2.3%); for *S pneumoniae* in 4/177 samples (2.3%); for *M catarrhalis* in 1/177 samples (0.6%).

Effect of typical bacteria on lung function in stable state and at exacerbation

Higher bacterial load was significantly correlated to disease severity in the stable state (FEV_1 % predicted); r=−0.299; p=0.033 (figure 5). In a multivariate analysis, the relationship between FEV_1 and airway bacterial load remains significant after allowance of either age (p=0.036) or smoking status (p=0.046), although it just misses statistical significance when both age and smoking status are included in the model (p=0.057).

Percentage (±SD) change in FEV_1 (from stable state) with *H influenzae*-associated exacerbations (n=10) was −17.4% (±15.8%), and in exacerbations which had no *H influenzae* presence (n=47), the change was −5.9% (±20.0%). This difference was not statistically significant (p=0.09).

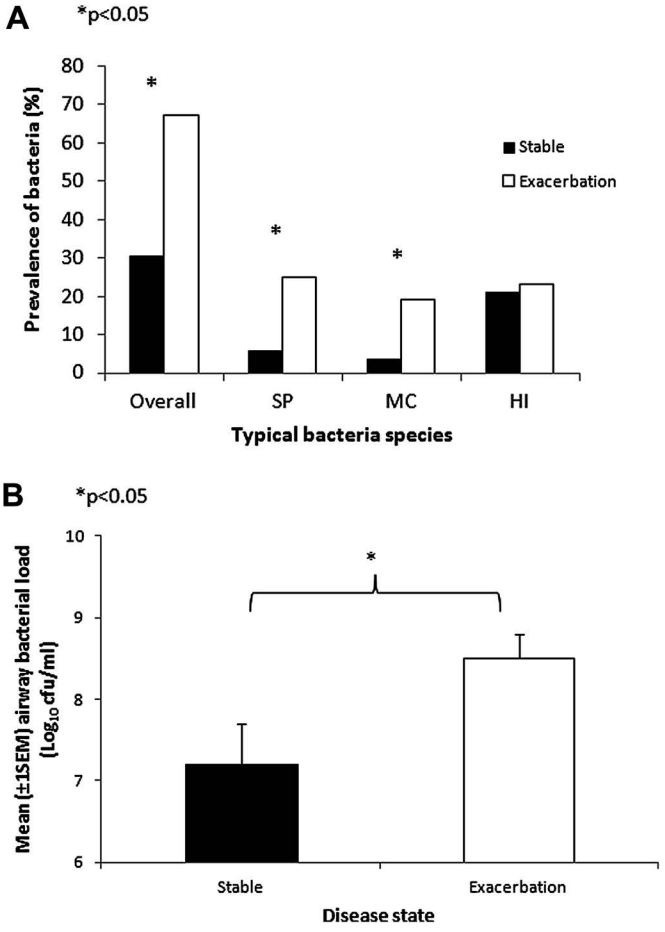

Figure 3 (A) Prevalence of typical airway bacteria in paired samples from COPD patients at both stable and exacerbation (n=65). (B) Overall bacterial load at stable state and exacerbation, in patients positive for typical airway bacteria at both states (n=12). Species: SP = *Streptococcus pneumoniae*; MC = *Moraxella catarrhalis*, HI = *Haemophilus influenzae*.

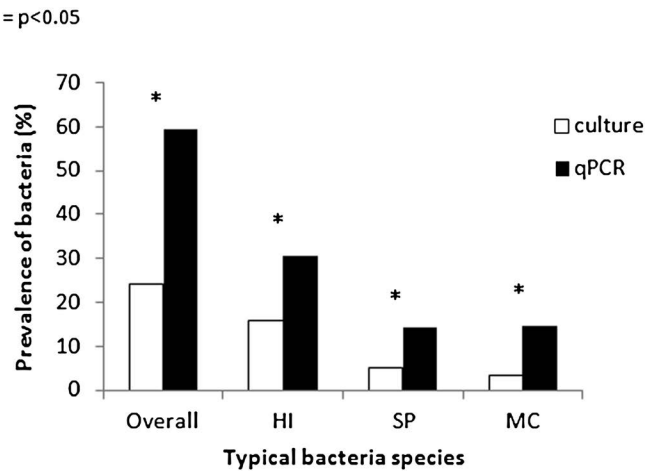

Figure 4 Comparison of culture and qPCR detection of three typical airway bacteria in sputa from 177 COPD patients (105 stable, 72 exacerbation). Species: HI = *Haemophilus influenzae*, SP = *Streptococcus pneumoniae*; MC = *Moraxella catarrhalis*.

Airway bacterial load association with inhaled corticosteroid usage

Forty-seven (92.2%) patients who had bacteria detected in the stable state had inhaled corticosteroid usage recorded. Median (IQR) beclomethasone-equivalent dosage was 2000 (640–2000) μg daily. In a univariate analysis, higher airway bacterial load was also significantly correlated to higher inhaled corticosteroid dosage (corrected for beclomethasone equivalence);

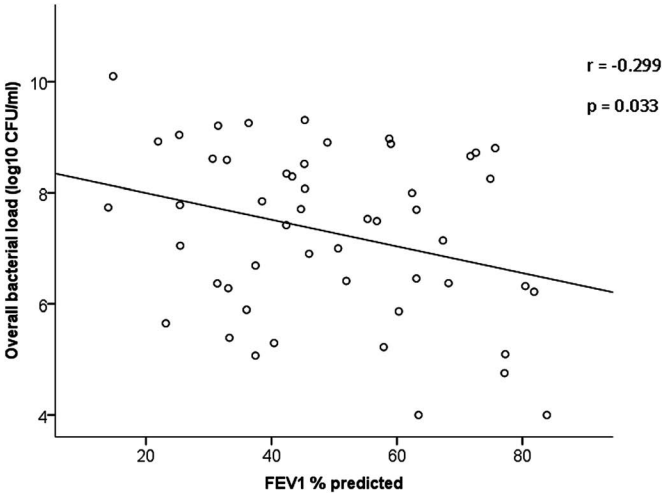

Figure 5 Association of bacterial load with FEV_1 in stable colonised patients (n=51). This illustrates the association of lower FEV_1 status in patients with higher bacterial loads. Data with FEV_1% predicted >80% were from patients who had FEV_1% predicted <80% at recruitment.

r=0.382; p=0.008. This relationship remains significant in a multivariate analysis including age, smoking status and FEV_1 % predicted (p=0.022).

Systemic inflammation is related to airway bacterial presence during exacerbations

Levels of CRP, a marker of systemic inflammation, were significantly related to presence of typical airway bacteria at exacerbation. CRP was measured in 119 exacerbation samples. Exacerbations with bacterial detection (n=58) showed significantly higher mean CRP compared with exacerbations with no bacterial detection (n=61); 35.0 versus 25.1 mg/l, p=0.032.

Analysis of COPD patient samples for atypical bacteria

A total of 176 samples, from 80 patients, were investigated for presence of the atypical bacteria (*C pneumoniae, L pneumophila, M pneumoniae*). Of these, 97 were stable samples, and 79 were exacerbation samples. Five samples were positive for *L pneumophila* (four stable, one exacerbation), and one stable sample was positive for *M pneumoniae*. No relationship was observed between atypical bacterial load and clinical outcome: Mean bacterial load for *L pneumophila* was $10^{4.4(\pm 0.4)}$, and for the *M pneumoniae* sample it was $10^{7.4}$ cfu/ml.

DISCUSSION

This is the largest study, to date, to use molecular techniques for detection of airway bacteria, and also the first to complimentarily evaluate typical and atypical bacteria in a well-characterised cohort of COPD patients. We have explored the relationship between airway bacterial prevalence and load in stable COPD patients and at exacerbations. At exacerbation, there was a marked increase in bacterial load in colonised patients, with an absolute increase in the region of 20-fold being identified in positive samples. We have conclusively demonstrated that qPCR is more discriminatory than culture at detecting typical airway bacteria. The role of atypical pathogens in COPD is minor, having been detected at low prevalence and load in very few samples.

We have found that in the stable state, there is a total prevalence rate for the three typical bacteria—analysed together—of 44.2%. *H influenzae* was the most prevalent species identified. *H influenzae* is also the most prevalent species in culture-based studies.[4 20] The prevalence rate using qPCR in this study was higher than prevalence seen in such culture studies. This work also highlights the association of airway bacteria with a poorer disease outcome in COPD and supports our previous findings, that increased airways inflammation may relate to airway bacterial colonisation[5] and is associated with faster FEV_1 decline.[8] In the stable state, we found significant correlation between higher bacterial load and more severe airflow limitation, independent of age or smoking status.

One previous study compared detection frequency of typical airway bacteria by qPCR and routine microbiological culture in COPD patients evaluating 30 samples.[21] In a larger dataset, we have now confirmed that qPCR is significantly more discriminatory than culture at detecting each of these three main airway bacteria. It is known that *H influenzae* is able to persist intracellularly in the respiratory tract, with possible resultant culture-negative sputum.[22 23] There were some instances in this study where a sample was culture-positive and PCR-negative, and there are a number of potential explanations for this, including technical issues with the primer targets, operator error or sampling, although the rates of such culture-positive PCR-negative samples were actually very low in our study. qPCR is, therefore, the most appropriate method to determine bacterial prevalence and load, with little additional sensitivity to be gained from running both methods in tandem.

The advantage of qPCR is that it gives a higher rate of detection than culture. Using this technique, this study demonstrates that exacerbations in COPD are characteristically associated with both increased prevalence and load of typical bacteria. This confirms previous data suggesting that the increase in load and acquisition of bacteria seen at exacerbation is driving such exacerbations.[24] Microbiological culture is, however, useful in other aspects of bacteriology, such as in the identification of antibiotic resistance, and so should not be wholly discounted. One limitation of PCR microbiological technique is that they can detect both viable and non-viable bacteria, while culture detects viable bacteria alone. However, to our knowledge, no studies have shown non-viable bacteria to be present at levels of 10^4 cfu/ml (the lower limit of detection for our PCR assays). Alveolar macrophages are responsible for clearance of DNA from the lungs, and so it is unlikely that DNA from lysed cells would persist for long periods.

It has also been previously demonstrated that airway bacterial presence is associated with increases in inflammatory cytokines.[7 25 26] We have found that bacterial presence at exacerbation detected by PCR is significantly associated with higher systemic inflammation using serum CRP. We also found a trend to increased fall in FEV_1 with HI-associated exacerbations, probably due to increased airway inflammation, and this is consistent with our previous data.[24] Many exacerbations of COPD also have a viral aetiology, and prior studies have demonstrated that exacerbations involving coinfection with bacteria and viruses are associated with a more impaired lung function.[24 27]

We also conclusively quantified typical airway bacteria in a cohort of COPD patients, with a concomitant illustration of significant increases in prevalence and load of three of the most commonly isolated bacteria seen in such subjects, at exacerbation. This provides further support to previous studies which hypothesise that alterations in 'normal' bacterial colonisation may trigger acute exacerbations of COPD.[28] This study has not examined the strain-switching hypothesis previously put forward.[29] However, strain acquisition is associated with an increase in bacterial load. Further work is needed to explore whether differences in stable and exacerbation loads are related to the same strain or different strains of specific bacterial species.

For the first time, we have also shown a significant relationship between airway bacterial load and inhaled corticosteroid dosage at the time of sampling in stable patients; this effect was independent of age, smoking status and disease severity. Findings from the TORCH and INSPIRE randomised controlled trials have indicated that inhaled corticosteroid use is related to increased frequency of developing pneumonia in COPD patients.[30–32] Our data suggests that the increase in airway bacterial colonisation may play a part in increasing susceptibility to pneumonia in COPD.

This study was limited to the three most commonly detected organisms, and it is probable that other organisms also contribute to the microbiome. However, recent detailed studies in small numbers of subjects have identified only the presence of commensals in addition to the common bacteria we studied, and it is unlikely that other bacteria are playing a significant role.[28 33 34] *P aeruginosa* was not studied in this analysis as its culture detection rate was very low in the London COPD cohort (<1%). It is difficult to differentiate between those strains that were infective and those which were colonising, due to the fact

that colonising strains are very likely to also appear at exacerbation. The different methodological approaches required are beyond the scope of this study.

The potential role of atypical bacteria in COPD was important to investigate with qPCR, as they are characteristically difficult to culture, and if found at high prevalence or load, could have major implications for therapy. A previous study found little evidence of atypical bacteria colonisation in a qPCR analysis of 248 sputum samples from COPD patients, with just one sample positive for *Legionella* spp.[11] The current study investigated the rate of these bacteria in COPD patients and found low prevalence, with one exacerbation sample (1.3%) identified with presence of *L pneumophila*. Atypical bacteria are, therefore, not major pathogens in COPD, either in the stable state or at exacerbation, and do not need specific therapy.

This study shows that at COPD exacerbations, airway bacterial prevalence and load increase significantly, and are an important aetiological factor driving the event. The presence of increased total bacterial load in the stable state and relationship to disease progression suggests that targeted antibiotic therapy for colonised patients may be beneficial. qPCR is more discriminatory than culture, identifying higher airway bacterial prevalence, and thus, should have an important role in routine clinical microbiological practice.

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Contributors Study design: DSG, GCD, TDM, JAW; sputum collection and processing: ARCP, AJM, JJPG; data analysis: DSG, GCD; DNA extraction: DSG, SJT; PCR: DSG; manuscript drafting & revision: DSG, ARCP, AJM, GCD, TDM, JAW; funding: JAW.

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Competing interests None.

Ethics approval Ethics approval was provide by Royal Free Research Ethics Committee.

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Changes in prevalence and load of airway bacteria using quantitative PCR in stable and exacerbated COPD

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interventional trials. Participants in this CF study had a field-based estimate of exercise capacity and objective measurement of physical activity at each study visit.

Methods Participants wore a pedometer for 7 days prior to each visit. At each visit, London patients performed a standard incremental shuttle-walk test and Edinburgh patients a modified shuttle test (in which running was allowed). Data are expressed as mean (SD).

Results Data were analysed from 192 patients over 648 visits. Age at enrolment was 24 (11.9) years (London) and 20.8 (9.9) (Edinburgh) (p=0.052); FEV_1 was 67 (17.7)% and 79 (19.5)% for each site respectively (p<0.001). Daily step count at visit 1 was 7491 (2887) in London and 8872 (4089) in Edinburgh (p=0.04) and this difference persisted across subsequent visits. The coefficient of variation (CV) in step counts between visits was 21.3%. Number of shuttles completed in London was 61 (15), and Edinburgh 90 (33) with no trend over the four visits (CV=10 and 16% respectively). In Edinburgh there was a correlation between mean step count and the number of completed shuttles (r=0.46, p<0.001). Step count from both sites, and the number of shuttles completed in Edinburgh, correlated with FEV_1 % predicted (r=0.24, p<0.001 and r=0.27, p<0.001 respectively) and with age (r=−0.28, p<0.001 and r=−0.30, p<0.001 respectively). Such correlations were either weaker or not observed in London, however, in this group, number of shuttles correlated with height (r=0.51, p<0.001).

Conclusions No changes were detected in exercise capacity or daily activity levels over time. Between site differences were observed in both measures; however, these populations also differ in age and FEV_1. The modified shuttle test performed in Edinburgh appeared to better correlate with clinical markers than the standard incremental shuttle test performed in London, and is independent of height. We believe that testing exercise capacity is important in CF and we plan to investigate the other testing methods in the run up to our Multi Dose Gene Therapy Trial.

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COPD: sputum and exacerbations

P110 QUANTITATIVE PCR-BASED DETECTION AND QUANTIFICATION OF ATYPICAL BACTERIA AT BASELINE AND EXACERBATION OF COPD

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Introduction Airway bacterial infections are associated with exacerbations of COPD. The potential role of atypical bacteria as a trigger for exacerbations is not well understood. Atypical bacteria such as *Chlamydophila pneumoniae* (CP), *Legionella pneumophila* (LP) and *Mycoplasma pneumoniae* (MP) are difficult to culture as they are intracellular pathogens. LP can be detected by urinary antigen, and serology can be performed for MP, but these techniques give no indication as to the atypical bacterial load. Quantitative PCR (qPCR) offers an alternative approach to identification and quantification of bacteria in sputum.

Methods Multiplex qPCR was used to detect and quantify CP, LP and MP in 238 samples prospectively collected from 87 patients in the London COPD Cohort: mean (±SD) age 71.4 (±8.1); predicted FEV_1 43.4% (±17.5%); male gender 47.9%; current smoker 49.2%. Baseline (n=104), exacerbation (n=95), and follow-up (n=39) samples were tested: Baseline was defined as at least 6-weeks without exacerbation, and exacerbation was defined as 2 consecutive days of two symptoms (Anthonisen criteria), at least one of which is a major symptom (dyspnoea; sputum purulence; sputum volume). Follow-up involved taking samples 2 or 5 weeks post-exacerbation onset. Using a qPCR developed by our clinical diagnostic service, the CP, MP and LP gene targets were RNA-polymerase β-chain; P1 adhesin protein; and MIP respectively. Routine microbiological analysis was also performed on these samples.

Results No samples were positive for the atypical organisms using culture. With qPCR analysis 6/238 samples (six separate patients) were positive for LP (2.5%), four at baseline and two at exacerbation/follow-up. One baseline sample was positive for MP (0.42%), and no samples were positive for CP. Atypical bacteria were present at 0.83% of exacerbations. Median (IQR) bacterial load was $4.3x10^4$ cfu ml^{-1} ($2.0x10^4$–$8.55x10^4$) for LP PCR-positive samples; the MP-positive sample load was $2.64x10^7$ cfu ml^{-1}.

Conclusion Quantitative PCR was more sensitive and informative than standard microbiological culture for the detection of atypical bacteria. Atypical bacteria in sputum were detected at very few exacerbations of COPD; moreover, when they were detected by qPCR, the load was low, indicating little or no significance in the aetiology of these events.

P111 FTIR SPECTROSCOPIC PROFILING OF COPD SPUTUM: IDENTIFICATION OF DISTINCT SPECTRAL SIGNATURES AND CORRELATION TO COPD STATUS

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COPD remains common and complex with huge costs on the NHS and disruption to patients' daily lives. This presents a challenge for an effective, non-invasive test to enable determination and monitoring of COPD status. Fourier transform infrared (FTIR) spectroscopy for sputum profiling is timely. FTIR identifies and measures chemical bond vibrations within functional groups in complex biological mixtures by producing infrared absorption spectra. Identifying COPD-relevant spectra in sputum could provide sensitive rapid information on status and exacerbations. To address this, we randomly recruited 102 patients independent of severity. Sputum was collected at initial visit and in each of subsequent 2 weeks. Dyspnoea and sputum scores were obtained; FEV_1, serum CRP and exhaled NO measured; any intervening chest infection or treatment change documented. Patients was stratified by FEV_1; 26 patients had mild COPD ($FEV_1 \geq 80\%$); 41 moderate (FEV_1 49–79%); 35 severe ($FEV_1 \leq 50\%$). FTIR was performed using an Alpha-T spectrometer (Bruker UK); transmission mode in 4000 to 900 cm^{-1} region; 4 cm^{-1} resolution. All COPD sputa gave reproducible biological IR spectra with distinct signatures in 5 key regions at 3300–3280 cm^{-1} (assigned as amide A), 3000–2800 cm^{-1}, 1660–1600 cm^{-1} (Amide I), 1560–1520 cm^{-1} (Amide II) and 1180–1000 cm^{-1} (glycoproteins). Multivariate analysis showed significant correlation between spectral profiles and FEV_1 and smoking habit. The accuracy of differentiating mild from severe COPD was consistently greater than 70% (AUC under ROC curve); specifically we observed peak shifts in Amide A towards 3300 cm^{-1} as COPD worsened (p<0.001). Also 68/102 patients exhibited a clear band around 2060 cm^{-1}; the remaining 34 showed no band in this region. There was a significant association (p=0.012) between peak presence and COPD severity; with mild COPD patients more likely to have a peak, and severe sufferers having 2060 cm^{-1} signal absence. 35/102 patients had exacerbations during the study. Separate spectral analysis showed significant increase in glycoprotein max peak during COPD exacerbation (p<0.03). This study has important implications for future near-patient COPD monitoring. FTIR

P110 Quantitative PCR-based detection and quantification of atypical bacteria at baseline and exacerbation of COPD

D S Garcha, S J Thurston, A R C Patel, et al.

Thorax 2010 65: A124
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Quantitative Pcr-Based Detection Of Typical And Atypical Bacterial Strains In COPD Patients And Relationship To Culture

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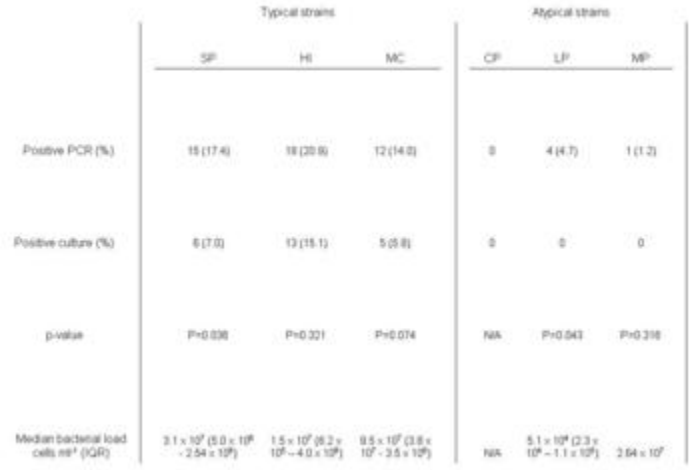
Rationale: Bacterial infections are common in chronic obstructive pulmonary disease (COPD). Typical bacterial species include Streptococcus pneumoniae (SP), Haemophilus influenzae (HI) and Moraxella catarrhalis (MC). The potential role of atypical bacteria such as Chlamydophila pneumoniae (CP), Legionella pneumophila (LP) and Mycoplasma pneumoniae (MP) has not been investigated in detail, largely due to difficulty culturing. Confirmation of infection for MP is by serology and LP is by detection of urinary antigen, but these techniques give no indication of bacterial load. We examined quantitative real time PCR (qPCR) as an alternative approach to identification and quantification of typical and atypical bacterial species in sputum.

Methods: Multiplex qPCR was used to detect and quantify SP, HI, MC, CP, LP and MP in 86 sputum samples prospectively collected from 52 patients in the London COPD cohort. The samples were divided for PCR and routine microbiological culture. Two multiplex PCRs were used to test for the typical and atypical gene targets. SP, HI and MC gene targets were Spn9082; Haemophilus influenzae P4 lipoprotein gene; and copB outer-membrane-protein gene, respectively. CP, MP and LP gene targets were RNA-polymerase β-chain; P1 adhesin protein; and macrophage infectivity potentiator respectively.

Results: Patient characteristics were: mean (±SD) age 71.8 (±8.1) years; predicted FEV_1 41.4% (±15.6%); male gender 73.3%; current smoker 25.6%. Culture analysis detected a total of 24 positive samples in a total of 22 patients (27.9% of total) from compared to 50 positive samples in 35 patients (58.1% of total) using qPCR ($p<0.001$). For individual species, qPCR detected a significantly greater proportion of SP and LP (Table 1). Mean (±SD) bacterial loads for the typical bacteria were not significantly different from one another: SP (7.4±1.4 $\log_{10}$ cells ml^{-1}) Vs HI (7.3±1.6) Vs MC (7.9±1.2). The bacterial load of LP (4.37±0.4) was found to be significantly lower compared with each of the three typical bacteria ($p<0.001$).

Comparison of bacterial strains detected by qPCR and by conventional microbiological culture



	Typical strains			Atypical strains		
	SP	HI	MC	CP	LP	MP
Positive PCR (%)	15 (17.4)	18 (20.9)	12 (14.0)	0	4 (4.7)	1 (1.2)
Positive culture (%)	6 (7.0)	13 (15.1)	5 (5.8)	0	0	0
p-value	P=0.038	P=0.321	P=0.074	NA	P=0.043	P=0.318
Median bacterial load cells ml^{-1} (IQR)	3.1×10^7 (5.0×10^6 - 2.54×10^8)	1.5×10^7 (6.2×10^6 – 4.0×10^8)	9.5×10^6 (3.8×10^6 - 3.5×10^8)	NA	5.1×10^4 (2.3×10^4 – 1.1×10^5)	2.64×10^7

Median bacterial load given from qPCR analysis. n = 86 samples. Definition of abbreviations: SP = Streptococcus pneumoniae; HI = Haemophilus influenzae; MC = Moraxella catarrhalis; CP = Chlamydophila pneumoniae; LP = Legionella pneumophila; MP = Mycoplasma pneumoniae. Significant differences at p<0.05.

Conclusion: Quantitative PCR is able to identify and quantify more positive samples than standard microbiological culture. There was no significant difference between the mean bacterial loads of the typical bacteria, as identified by qPCR. Atypical bacteria are infrequently identified in COPD sputum samples at a relatively low load, indicating a low clinical relevance.

This abstract is funded by: Medical Research Council

Conclusions Molecular profiling identifies heterogeneity in the airway microbiome of COPD patients, with dominance of pathogens routinely identified at culture. However, a precise role for bacteria in COPD remains unclear.

S17 IMPACT OF COPD SEVERITY AND SPUTUM PRODUCTION ON ANTIBIOTIC RESISTANCE

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Introduction Bacterial infections are a well-known trigger for exacerbations of COPD. A variety of antibiotics are regularly prescribed for this group of patients but the risk and frequency of antibiotic resistance in the COPD population is less understood. Routine culture data can be evaluated to establish resistance prevalence and patterns.

Methods Culture data were collected from the sputum samples of 293 patients in the London COPD cohort over a period of 5 years (01/01/2006–31/12/2010) mean (±SD) months in study 28.4 (±19.9); age 69.9 years (±8.9); predicted FEV_1 47.8% (±16.5); male gender 58%; exacerbation samples 48.9%; sputum producers 77.5%. Identification of bacterial presence was established and where clinically indicated drug sensitivity tests (DSTs) were performed. A resistant sample was reported as any bacterial isolate resistant to at least one antimicrobial agent.

Results 92/293 (31.4%) patients had at least one bacteria positive sample over the study period. 87/92 (94.6%) patients had samples where DSTs were performed on bacteria positive samples. Resistance was observed in 69/87 (79.3%) patients. 30/293 (10.2%) patients were resistant to all samples where DSTs were performed. 227/293 (77.5%) of patients were sputum producers. There was no significant relationship between predicted FEV_1 and antibiotic resistance frequency in this cohort (X^2-test; p=0.577). Patients who were classified as regular sputum producers were more likely to exhibit resistance in culture positive bacteria (p=0.048).

Conclusion Results from this analysis conclude that an estimated 23.5% of COPD patients will develop resistance to an antimicrobial agent within 28.4 months of follow-up with sputum producers being at a higher risk. This study highlights the importance of investigating sputum samples with determination of resistance patterns. Information on resistance patterns and transmission of resistance in COPD can allow more appropriate and targeted antibiotic therapy for COPD exacerbations with improved outcomes.

S18 A COMPARISON OF PREVALENCE AND LOAD OF AIRWAY BACTERIA IN COPD PATIENTS WITH PAIRED STABLE AND EXACERBATION STATE SAMPLES

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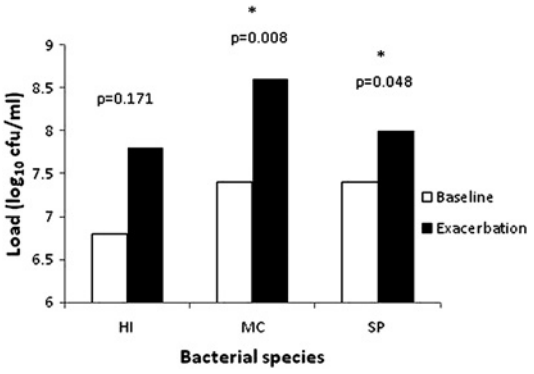
Introduction Airway bacterial infections are associated with COPD exacerbations. The most frequently identified bacteria in COPD are *Haemophilus influenzae* (HI), *Moraxella catarrhalis* (MC) and *Streptococcus pneumoniae* (SP) (Wilkinson *et al*, 2006), though studies have used culture techniques, with little data available on PCR methodology in airway infection. Using the London COPD cohort, we aimed to assess and quantify bacterial prevalence and load via quantitative PCR, in paired baseline and exacerbation sputum samples.

Methods Quantitative PCR was utilised, measuring prevalence and load on paired baseline and exacerbation samples, with baseline samples obtained within 1 year prior to its paired exacerbation. SP, HI and MC gene targets were Spn9082; *Haemophilus influenzae* P4 lipoprotein gene; copB outer-membrane-protein gene, respectively. The baseline state was defined as being at least 6 weeks after, and 2 weeks before, an exacerbation. Exacerbation was defined as two consecutive days of at least two increased symptoms (Anthonisen criteria), at least one of which is a major symptom (dyspnoea; sputum purulence; sputum volume).

Results Sixty-nine paired baseline and exacerbation sputum samples were obtained from 56 patients: mean (±SD) age 71.0 years (±8.4); predicted FEV_1 46.4% (±17.0); male gender 60.4%; current smoker 30.2%. Bacteria were detected at significantly higher rate at exacerbation, being seen in 36/69 (52.2%) exacerbations, and 19/69 (27.5%) baseline samples (χ^2-test; p=0.003). Mean bacterial load was significantly higher at exacerbation, with mean load of 8.3 (±1.1) $\log_{10}$ cfu/ml, compared with mean of 7.3 (±1.8) $\log_{10}$ cfu/ml at baseline (paired-samples t test; p<0.001), indicating a 10-fold overall-load increase at exacerbation. MC frequency increased significantly from 4.3% (3/69) at baseline to 17.4% (12/69) at exacerbation (p=0.014). Prevalence of HI (17.4% vs 26.1%) and SP (8.7% vs 20.3%) showed non-significant increases. Mean loads of SP and MC increased significantly from baseline to exacerbation (p=0.048; p=0.008, respectively).

Conclusion Prevalence and load of airway bacteria in COPD increases from baseline to exacerbation. This confirms that bacteria play an important role in exacerbation aetiology, implicating increasing bacterial load as a key underlying mechanism, and emphasises the importance of prompt antibiotic therapy at COPD exacerbation.



Abstract S18 Figure 1 Bacterial load at baseline and exacerbation of COPD as determined by quantitative PCR.

S19 MOLECULAR FINGERPRINTING AND METAGENOMIC ANALYSIS REVEALS A POLYMICROBIAL ELEMENT IN PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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Introduction and objectives Chronic obstructive pulmonary disease (COPD) patients commonly exhibit a multi-factorial pathology with neutrophilic inflammation and chronic obstructive bronchiolitis. COPD patients suffer episodes of pulmonary exacerbations. The role of bacteria in exacerbations has been investigated in COPD using culture-dependent techniques. Unlike cystic fibrosis (CF), there are few molecular studies describing the possibility of a

S18 A comparison of prevalence and load of airway bacteria in COPD patients with paired stable and exacerbation state samples

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Association Between Airway Bacterial Load And Clinical Parameters In Stable COPD And At Exacerbation

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Introduction:

Airway bacteria are associated with COPD exacerbations, with the typical bacteria Haemophilus influenzae (HI), Moraxella catarrhalis (MC) and Streptococcus pneumoniae (SP) frequently cultured. Quantitative PCR provides an accurate measurement of bacterial prevalence and load. We aimed to explore the relationship of bacterial load with FEV_1 status in stable COPD outpatients, and with sputum purulence at exacerbation.

Methods:

Real-time quantitative PCR was used to assess prevalence and load of HI, MC and SP. Spirometry was performed in clinic. Baseline was defined as being at least six weeks following, and two weeks before, an exacerbation. Exacerbation was defined as two consecutive days of two symptoms (Anthonisen criteria), on prospectively-collected diary cards, at least one of which is a major symptom (dyspnoea; sputum purulence; sputum volume), with the other being either a major or minor symptom (wheeze, cold, sore throat and cough).

SP, HI and MC gene targets were Spn9082; Haemophilus influenzae P4 lipoprotein gene; and copB outer-membrane-protein gene, respectively.

Results:

Characteristics for 134 COPD patients: mean (±SD) age 69.8 (±8.7) years; FEV_1 (L) 1.22 (±0.5), at 48.5% (±18.1) predicted FEV_1; FEV_1/FVC 0.46 (±0.1); male gender 64.2%; current smoker 28.4%. For stable-state and exacerbation, 242 and 131 samples were analysed, respectively. Sixty-five paired stable and exacerbation sputum samples were obtained from 52 of these patients: stable sample taken median (IQR) 100 (35-172) days prior to its reciprocal exacerbation.

In stable COPD, higher bacterial load (HI, MC, SP) was significantly related to an equivalent poorer FEV_1 status during stable state, shown in Figure 1 (r=-0.299; p=0.033). In patients colonised at both stable and exacerbation state, there was no significant correlation between total increase in load and change in FEV_1 at exacerbation (r=0.236; p=0.210). However, HI presence at exacerbation suggested a more severe fall in FEV_1 (-17.4±15.8%) compared to exacerbations without HI (-4.1±24.7%) although this did not reach statistical significance (p=0.086). Bacterial load trended towards being significantly higher in samples with sputum purulence against non-purulent (8.4±1.1 vs 7.8±1.4 $\log_{10}$ CFU/ml; p=0.071).

Conclusion:

We have shown that higher bacterial load in stable COPD patients is associated with poorer lung function. In patients harbouring lower airway bacteria during stable state, increase in load at exacerbation was not significantly associated with a fall in FEV_1. These findings implicate bacterial load at stable state as having a role in disease severity, and underscores the role of prompt antibiotic therapy.

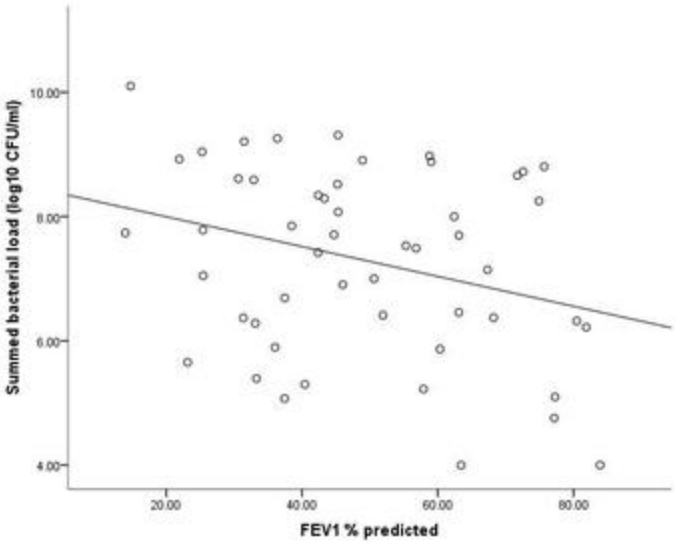

Figure 1. Association of bacterial load with FEV1 status in stable colonised patients (n=51). Figure 1 illustrates the association of higher sum bacterial load with poorer FEV1 status. Note that samples with FEV1% predicted >80% were from patients who had FEV1% predicted <80% at recruitment

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Bacterial prevalence and load during COPD exacerbation and recovery

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Bacteria are a common aetiological trigger of COPD exacerbations, with prevalence and load increasing from stable state (Garcha et al, *Thorax* 2011: 66;A11). We investigated prevalence and load of *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* during exacerbation recovery using quantitative PCR (qPCR).

We collected sputum from subjects in the London COPD cohort at exacerbation presentation (n=137), day 3 (n=41), 7 (n=53), 14 (n=56) and 35 post-exacerbation (n=35). All exacerbations were treated with ≥7 days of antibiotics (±oral steroids) and defined by our usual symptomatic criteria (Seemungal et al, *AJRCCM*, 1998).

Characteristics of 102 COPD patients: mean(SD) age 68.7(8.2) years; FEV_1 1.2(0.5)L, 48.6(24.8)% predicted. Bacterial prevalence was higher at exacerbation than Day 3 (50.4 vs 26.8%; p=0.008), Day 7 (28.3%; p=0.006) and Day 14 (30.4%; p=0.011) (Fig. 1). In recovery samples paired with bacteria-positive exacerbations (different patients at each time point), load [median (IQR) Log_{10} CFU/ml] was significantly higher at exacerbation compared with Day 3 (n=15): $10^{8.1(5.9\text{-}8.7)}$ vs $10^{0(0\text{-}4.0)}$, p=0.001; Day 7 (n=17): $10^{8.2(6.3\text{-}8.8)}$ vs $10^{4.2(0\text{-}6.6)}$, p=0.01; Day 14 (n=14): $10^{8.0(6.3\text{-}8.7)}$ vs $10^{4.4(0\text{-}6.6)}$, p=0.022; and Day 35 (n=15): $10^{7.7(5.7\text{-}9.3)}$ vs $10^{5.9(0\text{-}6.74)}$, p=0.047.

Bacterial prevalence and load detected by qPCR falls within 3 days of antibiotic therapy for COPD exacerbations, with load remaining low for at least 35 days post-onset.